STUDIES ON NEW ZEALAND MARINE NATURAL PRODUCTS

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Chemistry

at the

University of Canterbury

by

Rachel E. Lill

University of Canterbury

February, 1999

Abstract

The halichondrins, isolated from the Kaikoura sponge *Lissodendoryx* sp., are a series of polyether macrolides displaying potent *in vivo* antitumour activity. As such, they represent important leads as potential anticancer drugs. This research was focused on methodology that would enhance and complement the progress of halichondrins toward clinical trials.

A method was established for transforming 53-methoxyneoisohomohalichondrin B, an artefact of the methanol extraction method, to the more useful isohomohalichondrin B. A by-product of this acid treatment was identified as the C38 epimer of isohomohalichondrin B. The equilibrium character of the C38 epimerisation was studied.

The production of halichondrins by aquacultured *Lissodendoryx* sp. has been confirmed.

Haptens presenting the macrocyclic portion of the halichondrins were produced using three different linkers PMPI, PMSI and EMCH. The chemistry required to selectively oxidise the C26 methylene was established.

A polymer drug conjugate incorporating a halichondrin species has been prepared. This required the modification of homohalichondrin B to form an amino halichondrin.

Acknowledgments

I would like to express my gratitude to my supervisors, Dr John Blunt and Dr Murray Munro, for their encouragement and enthusiasm throughout the course of my time in the Marine Chemistry Group.

The friendly assistance of the technical staff is gratefully acknowledged. Special thanks go to Gill Ellis for performing the P388 murine leukemia assays, and to Bruce Clark for mass spectrometric analysis. I would also like to thank Lewis Pannell (NIDDK, NIH, USA) for additional mass spectrometric analysis and Dr Michael Boyd (NCI, NIH, USA) for biological assays. The assistance of Sarah Hickford in the isolation and purification of the halichondrins used in this research was much appreciated.

Thanks must also go to FRST and the NCI for their financial contributions towards this project. The contributions of the Royal Society, NZIC, PharmaMar (Spain), the Evans Fund and the Gordon Conference Committee toward my attendence at the 1998 Gordon Conference on Marine Natural Products, Ventura, CA, USA is gratefully acknowledged.

I would like to thank my fellow students for their friendship and support, especially David Stirling, Mike Stewart, Jo Hart, Sarah Hickford, Carolyn Coulter, Gill Nicholas, Susie Meade and Andy Phillips. Further thanks go to Andy for his thoughts on the synthetic aspects of my research.

A big thankyou to my parents for their ongoing support and diversionary garden expeditions, and to my grandparents for their keen interest in my progress. Clare, Tim, Mac and Theresa McLennan have been great providers of free meals and entertainment as my Christchurch family.

The biggest thankyou is reserved for Gavin (aka cook, dishwasher, computer consultant, bowling machine,...). Thank you Gavin for your constant support and friendship.

Table of Contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 6

Chapter 7

Abbreviations

Ac	acetyl
APT	attached proton test
Ar	aryl
Bz	benzoate
bpt	boiling point
°C	degrees Celsius
calcd	calculated
cm	centimetre(s)
conc	concentration
COSY	correlation spectroscopy
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet (spectral)
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
Et	ethyl
FAB	fast atom bombardment (in mass spectrometry)
g	gram(s)
GC	gas chromatography
HMBC	heteronuclear multiple bond coherrance (in NMR)
HPLC	high-performance liquid chromatography
HRFABMS	high-resolution fast atom bombardment mass spectrometry

Abbreviations

HSMQC	heteronuclear single and multiple quantum coherence (in NMR)
HSQC	heteronuclear single quantum coherrance (in NMR)
Hz	hertz
J	coupling constant (in NMR)
L	liter(s)
μ	micro
m	multiplet (spectral), milli
M	moles per liter
mol	mole(s)
MS	mass spectrometry
m/z	mass to charge ratio (in mass spectrometry)
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
ppm	parts per million (in NMR)
rt	room temperature
s	singlet (NMR); second(s)
t	triplet (spectra)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy (in NMR)
UV	ultraviolet

Chapter 1

Introduction

1.1 Natural Products as Potential Pharmaceuticals

Williams[1] defines a natural product as *a substance that has no known role in the internal economy of the producing organism.* It is suggested that the ability to produce a battery of secondary metabolites (natural products) which interact with other organisms has evolved as one facet of the organism's survival strategy. Furthermore, *all natural products have evolved under the pressure of natural selection to bind to specific receptors.*

It has been observed that natural products are common in organisms that lack an immune system.[1] The converse of this suggests that natural products will be sparse in organisms which possess an immune system. In the absence of an established immune system plants, microorganisms such as fungi and at least the most primitive invertebrates have to rely on chemical defences to aid their survival. It is these natural products produced as defence chemicals that offer a vast resource of potential pharmaceuticals. The best example of this resource of potential pharmaceuticals has been the sessile higher plants, from which a rich array of pharmaceuticals have been developed. Based on the currently accepted arguments[1] it can be suggested that these same plants are those that have evolved a range of metabolites as a chemical defence against parasites and predators.

The search for new and improved pharmaceuticals is ongoing. There is an estimated worldwide incidence of six million new cases of cancer every year.[2] Considering this

alongside the occurrence of AIDS, and the growing resistance of bacterial infections to present day antibiotics, the demand for new and efficacious pharmaceuticals is immense.

The unique, varied and structurally complex nature of secondary metabolites make them an invaluable resource in the search for novel bioactive agents.

1.2 Marine Natural Products

The majority of phyla and more than 90% of all classes of organisms are represented in the marine environment.[3] Marine organisms thus provide a rich resource for the discovery of novel biologically active compounds. It has been estimated that 72% of known life forms are from the Animal Kingdom.[4] The overwhelming number of animals belong to the two insect phyla. However, the balance of the Animal Kingdom, representing ~ 20% of all known life forms, are primarily aquatic, notably marine, in habitat. Of the twenty seven animal phyla, twenty six are found in the marine environment. This distribution of animal phyla in favour of mainly marine benthic environments is illustrated in Figure 1.2.1. By sampling marine animals one is sampling from the most biodiverse source on Earth.

The collection of marine organisms was limited to shore wading and shallow diving before the advent of SCUBA enabled greater access to the marine environment. This allowed collection of organisms down to depths of about 40 metres. Collection below this depth can only be achieved by dredging, which by its nature is non-targeted, or by using remote operated vehicles or specialist manned submersibles such as the Johnson Sealink.

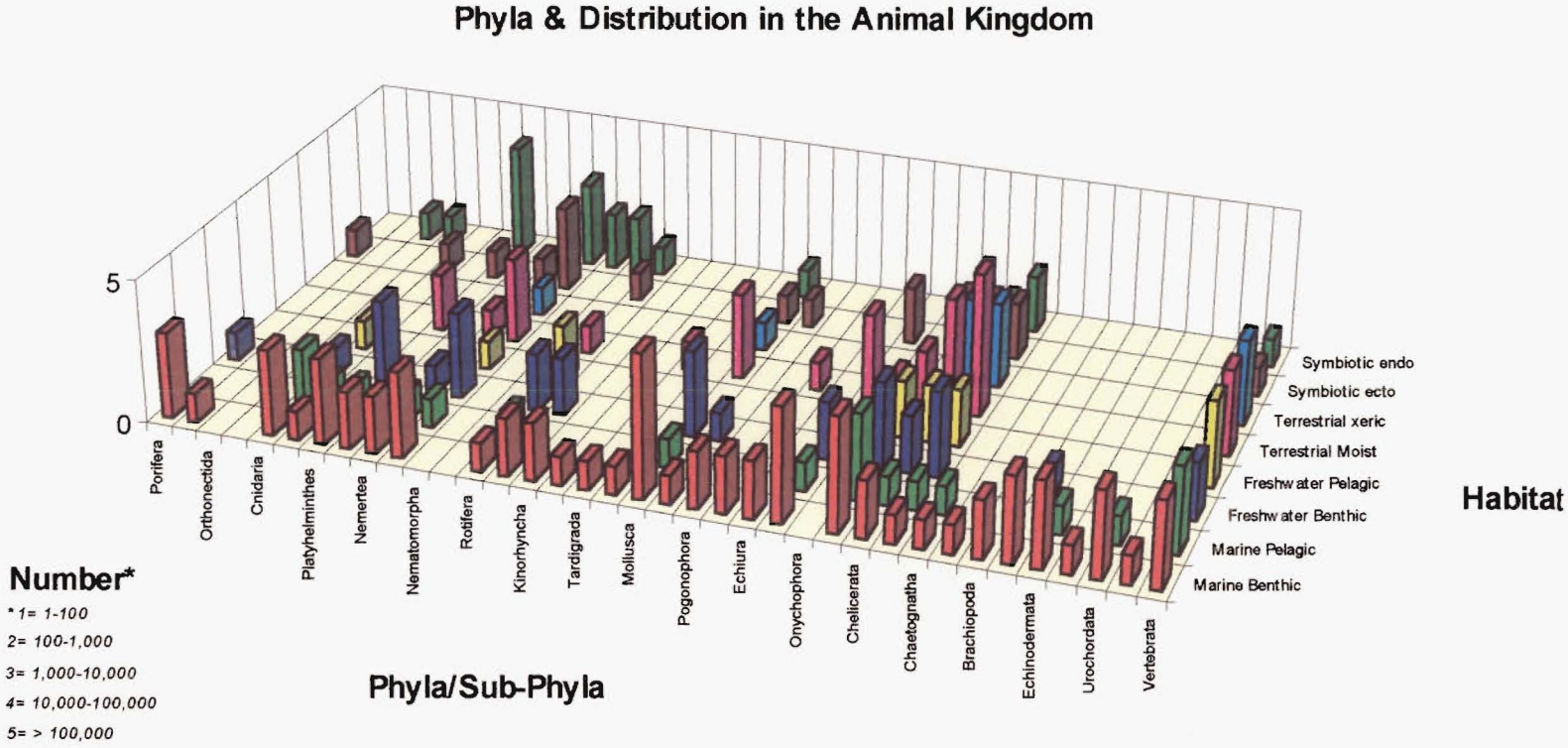


Figure 1.2.1 Distribution by Phylum in the Animal Kingdom

Data from the National Cancer Institute (NCI) in the USA, compiled by Dr Peter Murphy of the Australian Institute of Marine Sciences (AIMS) in 1992,[5] further illustrate the value in screening marine natural products for potential pharmaceutical leads. The number of anticancer leads from various sources in the NCI's *in vitro* preclinical antitumour drug screen are displayed in Figure 1.2.2. The high proportion of anticancer leads coming from marine animals relative to other sources is clearly illustrated. Australian results from AIMS[5] indicate more specifically that the marine invertebrate phylum Porifera (sponges) provides a rich source of cytotoxic marine natural products, with greater than 11% of specimens in this phylum displaying significant cytotoxicity.

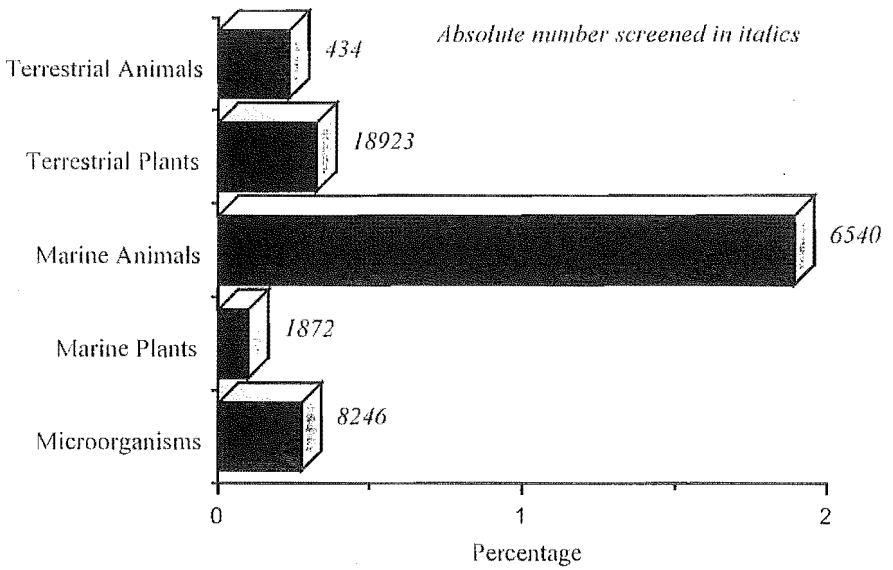
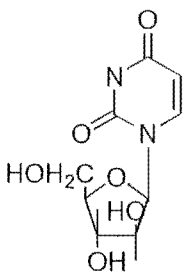


Figure 1.2.2 Percentage of Anticancer Leads with Significant Selective Cytotoxic Activity in the NCI Preclinical Antitumour Drug Discovery Screen.[5]

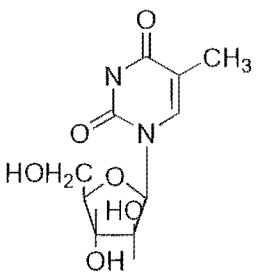
1.2.1 Bioactive Marine Natural Products

The ara-nucleosides spongouridine (**1.1**) and spongothymidine (**1.2**) isolated from the Caribbean sponge *Cryptothethia crypta* in the early 1950s by Bergman[6] were among the

first bioactive marine natural products to be characterised. The related cytosine arabinose (Ara-C) was eventually developed as a clinically useful anticancer drug. However the systematic investigation of marine environments as sources of novel biologically active agents only began in earnest in the mid 1970s.[7] A review of the marine natural products literature up to early 1986[8] reported 185 bioactive compounds. A 1993 review by Schmitz *et al* commented on an additional 400 compounds.[9] A survey of MarinLit[10] reveals that this trend has continued with 46% of all cytotoxic compounds in the database having been reported since 1993. This overview will focus solely on promising anticancer compounds isolated from marine organisms.

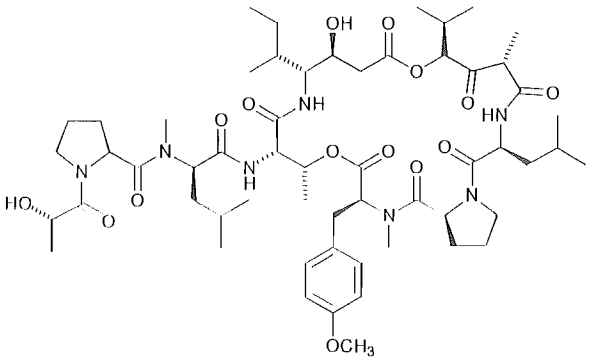


spongouridine (**1.1**)

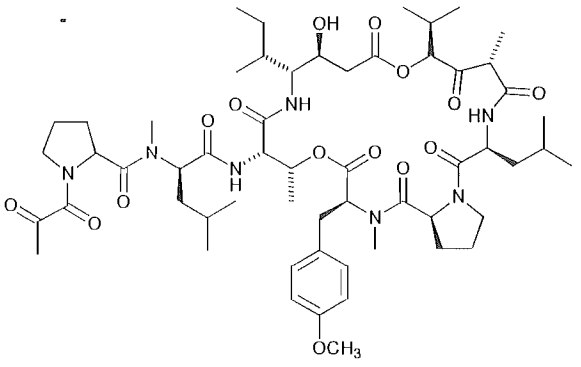


spongothymidine (**1.2**)

No compound isolated from a marine source has yet reached clinical use as an anticancer agent, although several are in various stages of clinical testing. Didemnin B (**1.3**), isolated from the tunicate *Tridemnum solidum*, was the first marine-derived compound to enter clinical trials.[11] Phase II clinical trials on didemnin B (**1.3**) were discontinued because of a failure to show reproducible activity against a range of tumours, while always exhibiting toxicity. A related compound, dehydrodidemnin B (**1.4**), isolated from the tunicate *Aplidium albicans*, is even more potent than didemnin B.[12] This depsipeptide is currently in Phase I clinical trials in the US and Europe.

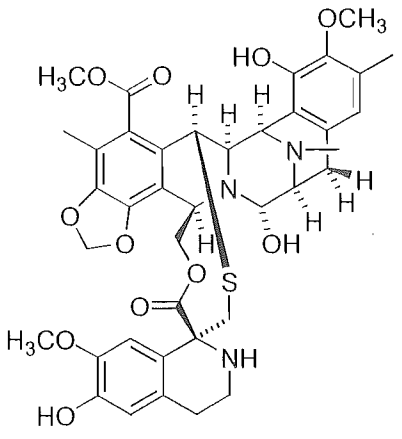


didemnin B (**1.3**)



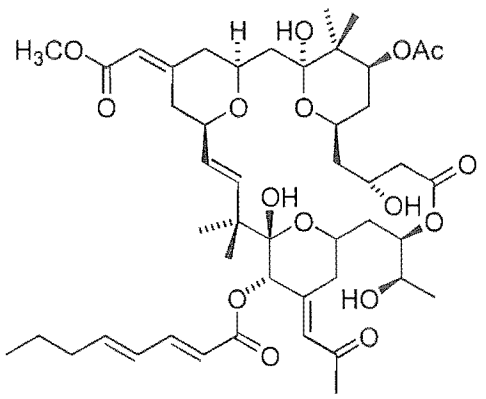
dehydrodidemnin B (**1.4**)

A number of ecteinascidins have been isolated from the tunicate *Ecteinascidia turbinata*[13] including ecteinascidin 743 (**1.5**).[14] This antimitotic agent exhibits significant *in vivo* activity against the murine B16 melanoma and human MX-1 breast carcinoma models and is currently in Phase II trials in the US and Europe.



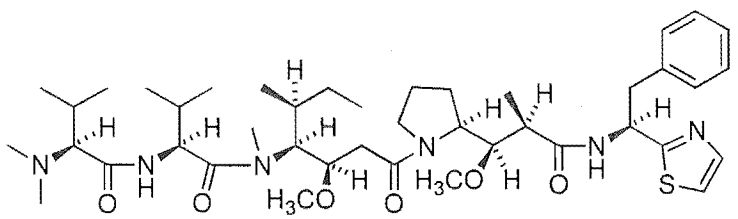
ecteinascidin 743 (**1.5**)

A protein kinase C modulator isolated from the bryozoan *Bugula neritina*, bryostatin 1 (**1.6**),[15] showed promising activity against melanoma in Phase I trials.[7] This compound progressed to Phase II trials but was withdrawn in late 1998.



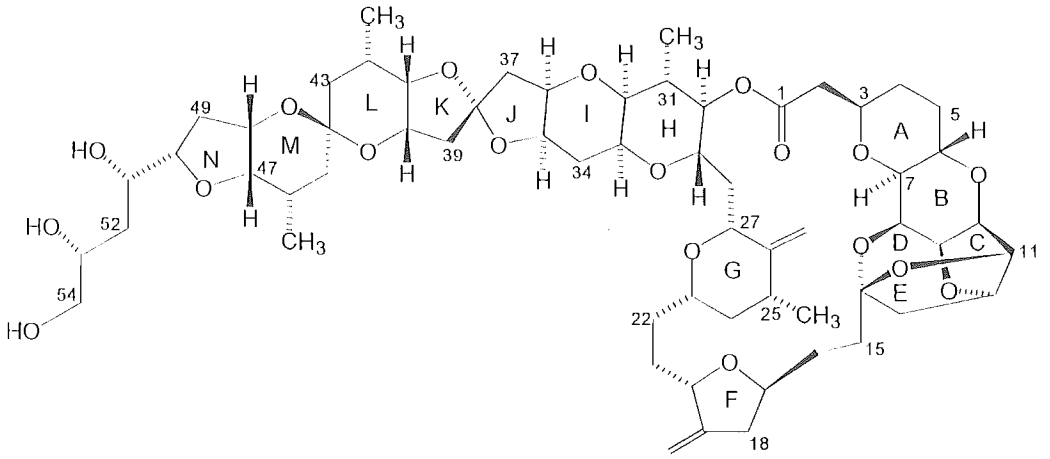
bryostatin 1 (**1.6**)

Dolastatin 10 (**1.7**) was originally isolated from the sea hare *Dolabella auricularia*.[16] The low yield (~ 1 mg/100 kg wet weight) of dolastatins from this organism hindered the development of **1.7**. However dolastatin 10 has since been synthesised,[17] and has progressed to Phase I clinical trials.

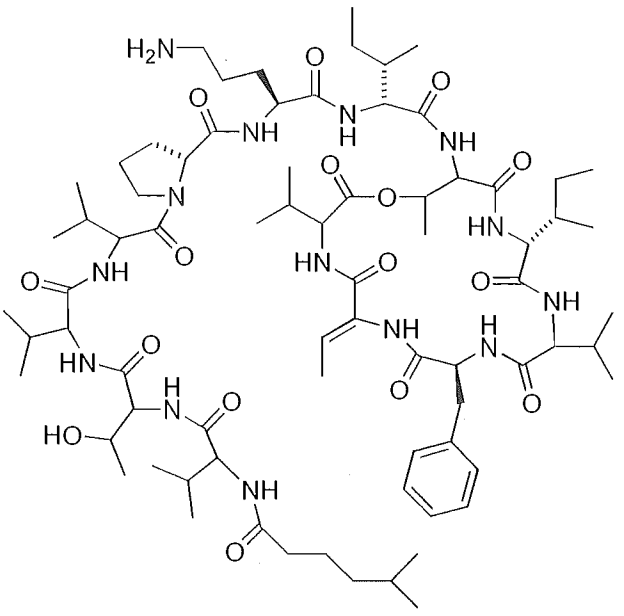


dolastatin 10 (**1.7**)

Halichondrin B (**1.8**), initially isolated from the sponge *Halichondria okadai* in 1985,[18] and kahalalide F (**1.9**),[19] isolated from a marine mollusc, are currently in preclinical development. Halichondrin B is an antimitotic agent, inhibiting the polymerisation of tubulin.



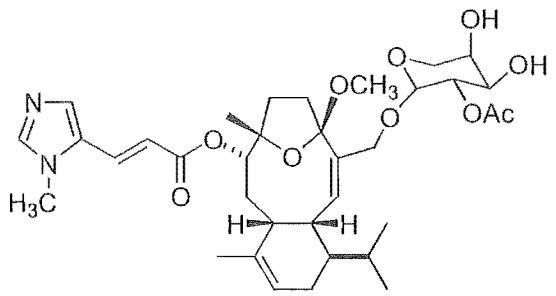
halichondrin B (**1.8**)



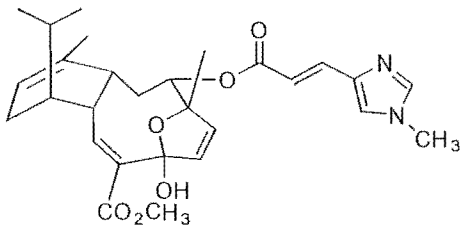
kahalalide F (**1.9**)

A number of other compounds exhibit promising *in vivo* anticancer activity. Eleutherobin (**1.10**), isolated from the Australian soft coral *Eleutherobia sp.*, stabilises microtubules preventing tubulin depolymerisation, and is cytotoxic to cancer cells with an IC_{50} similar to that of paclitaxel.[20] This compound has been synthesised by Nicolaou[21] and Danishefsky.[22] The sarcodictyins, isolated by Pietra[23] from a soft coral, are a related series of compounds that also act by stabilising microtubules. Sarcodictyin A (**1.11**) and B, and a library of analogues have been synthesised by Nicolaou.[24] A third compound, discodermolide (**1.12**), also possesses tubulin stabilisation properties.[25]

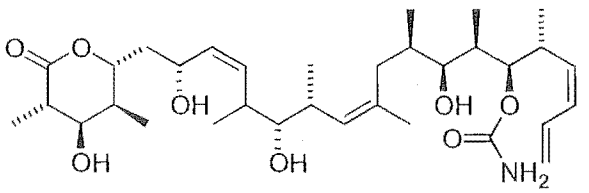
Discodermolide (**1.12**) was isolated from the deep water sponge *Discodermia dissoluta* by Gunasekera.[26]



eleutherobin (**1.10**)

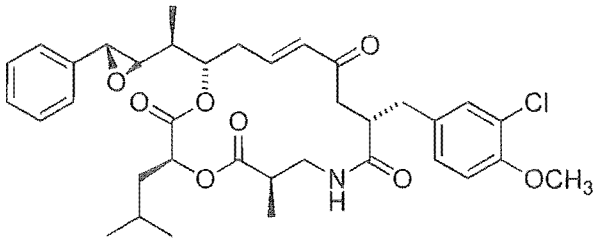


sarcodictyin A (**1.11**)

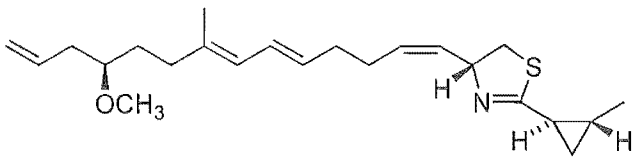


discodermolide (**1.12**)

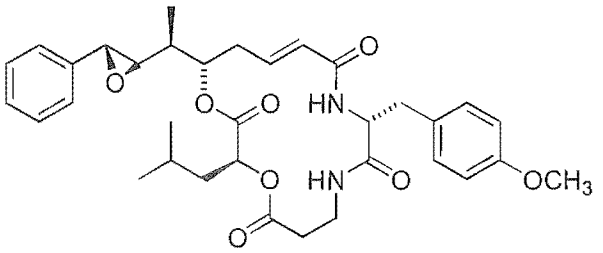
Isolated from the blue-green algae *Nostoc sp.* GSV 224, the cryptophycins[27] are a family of depsipeptides showing highly potent cytotoxic activity. The recently synthesised cryptophycin 1 (**1.13**)[28] acts as a microtubule poison. Interest in the compound has been heightened by the discovery that cryptophycin 1 (**1.13**) shows reduced susceptibility to the multidrug resistance pump, and shows no reduction of activity in a number of drug-resistant cell lines.[29] Further examples of antimitotic marine natural products include curacin A (**1.14**), isolated by Gerwick[30] from the blue-green alga *Lyngbya majuscula*, arenastatin A (**1.15**), isolated from the Okinawan sponge *Dysidea arenaria*,[31] and hemiasterlin (**1.16**), isolated from *Hemiasterella minor* by Kashman and coworkers.[32]



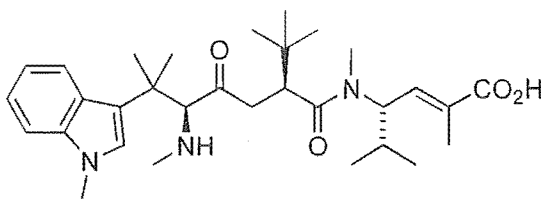
cryptophycin 1 (**1.13**)



curacin A (**1.14**)

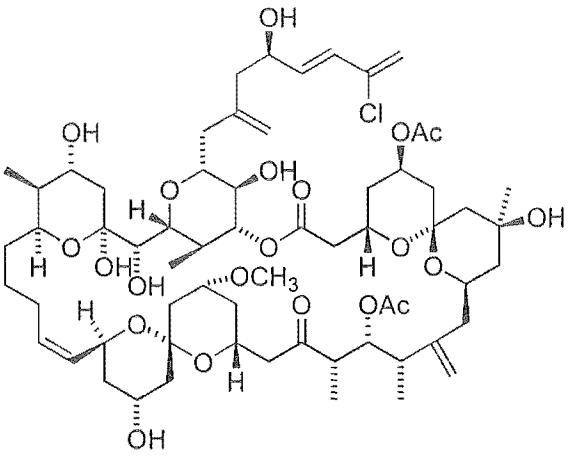


arenastatin A (**1.15**)



hemiasterlin (**1.16**)

The spongistatins, exemplified by spongistatin 1 (**1.17**), were reported independently by three research groups in 1993,[33] and have since been synthesised by several groups.[34] The spongistatins are potent antimitotic agents, acting at the vinca domain.



spongistatin 1 (**1.17**)

1.2.2 Sponges[35]

Sponges are members of the phylum Porifera ("pore-bearing"). They seem to have travelled an independent evolutionary route from other members of the animal kingdom and as such are sometimes placed in a subkingdom of their own, the Parazoa ("beside the animals"). Sponges differ from all other groups of invertebrates which occupy similar ecological niches in that they maintain an almost protozoan independence for their constituent cells, while at the same time ensuring that the entire cell mass pumps sufficient water to effect all essential exchanges.[36] There are approximately ten thousand species of sponge, grouped into four classes according to their skeletal structure. Sponges are benthic organisms that can be found in any situation, from upper intertidal to hadal depths (down to 8600m), in fresh and brackish water, in caves and in full illumination.[37]

Adult sponges are sessile hollow filter feeders. Water containing food particles is drawn into the internal cavity of the sponge, the atrium, through numerous perforations known as incurrent pores. The water is strained of food particles and leaves the atrium through one or more large excurrent pores (osculum). The flow of water is maintained by a combination of the sucking effects of the flow of local currents across the osculum and

by the beating of flagella protruding from the collars of choanocytes which line the interior cavity of the sponge.

The sessile nature of sponges makes them a prime target for fouling or overgrowth by other organisms. Their continued existence points to the evolution of a complex chemical defense.

1.2.3 The Marine Chemistry Group

The Marine Chemistry Group at the University of Canterbury was formed in 1975. The prime objective of the Group was the isolation and identification of biologically active marine natural products.[38]

The Group has an extensive and ongoing collection of New Zealand marine organisms. Extracts of two gram sub-samples of every organism collected are screened in the Marine Chemistry Group's in-house antitumour, antiviral and antimicrobial assays. Organisms producing extracts that exhibit favourable biological activity are extracted on a larger scale. These bulk extracts undergo bioactivity-guided chromatographic fractionation to isolate the active components. The success of this approach is illustrated in Section 1.2.3.2.

1.2.3.1 The P388 Antitumour Assay

The *in vitro* "antitumour" assay uses the P388 cell line (murine leukemia cells) and is up to a hundred times more sensitive to cytotoxicity effects than the antiviral BSC-1 cell line. The P388 assay is therefore a cytotoxicity-based assay against a specific leukemia cell type. The IC_{50} result obtained from this assay represents the concentration of the test compound at which the number of viable cells is reduced by fifty per cent relative to the control.

A two-fold dilution series of the sample of interest is incubated with P388 cells and MTT, a yellow tetrazolium salt, in small plastic wells. After seventy-two hours of incubation the concentration of the sample required to reduce the number of viable cells by fifty per cent relative to a control (P388 cells, MTT, media and solvent) is calculated. The mitochondria of viable leukemia cells reduce the yellow dye MTT to a purple formazan derivative.[39] By measuring the light absorbance at 540 nm in each well, a direct quantification of formazan formation and therefore the number of viable cells is able to be determined. The absorbance is expressed as a percentage cell viability relative to the control, and is plotted against the logarithm of the sample concentration in the well to generate a sample concentration *vs* cell viability curve. The antilogarithm of the concentration producing a fifty per cent reduction in the number of viable cells gives the IC_{50} result, which is usually expressed in units of ng/mL.

The P388 cell line represents a rapidly dividing cell type, thus its efficacy in finding products with activity against the predominantly occurring slow growing solid tumours such as lung, colon, breast, skin and kidney cancers is strictly limited.[40] However, it is important to consider that a P388-active compound may still display selective activity against these types of tumour.

1.2.3.2 The National Cancer Institute (NCI) Anticancer Screen

An *in vivo* P388 murine leukemia screen was employed as the primary anticancer screen at the NCI from 1975 to 1985.[41] In 1985 the NCI began investigating a disease-orientated *in vitro* screen using a wide variety of human tumour cell lines because of concern that the P388 screen was only highlighting agents with activity against leukemia-type cancers. Routine operation of the *in vitro* screen began in 1990. In the primary screening, each compound is tested over a broad concentration range (five 10-fold dilutions starting from 10^{-4} M) against each of the sixty cell lines in the panel. Three different target responses may be chosen to quantitate the data: by the GI_{50}, LC_{50} or TGI response. The GI_{50} represents the drug concentration producing an apparent

fifty per cent decrease in the number of tumour cells relative to an appropriate non-drug control at the end of the drug incubation.[42] The LC_{50} is defined as the concentration of drug producing an apparent fifty percent reduction in the number of tumour cells in reference to an appropriate non-drug control at the start of the incubation. The TGI is the concentration at which the number of tumour cells remaining at the end of the incubation is the same as at the beginning of the incubation.

A mean-graph format can be constructed to display the data. This graph displays horizontal bars for each individual cell line for any of the desired target responses (for example, the negative log_{10}TGI results) relative to the averaged response (negative log_{10}TGI) over all cell lines. The mean graph therefore provides a characteristic "fingerprint" for a given compound, depicting individual cell lines that are proportionally more sensitive than average (bars to the right of the average response) or less sensitive than the average (bars to the left of the average response). The mean graph for norhomohalichondrin B (**4.17**) is displayed in Figure 1.2.3.

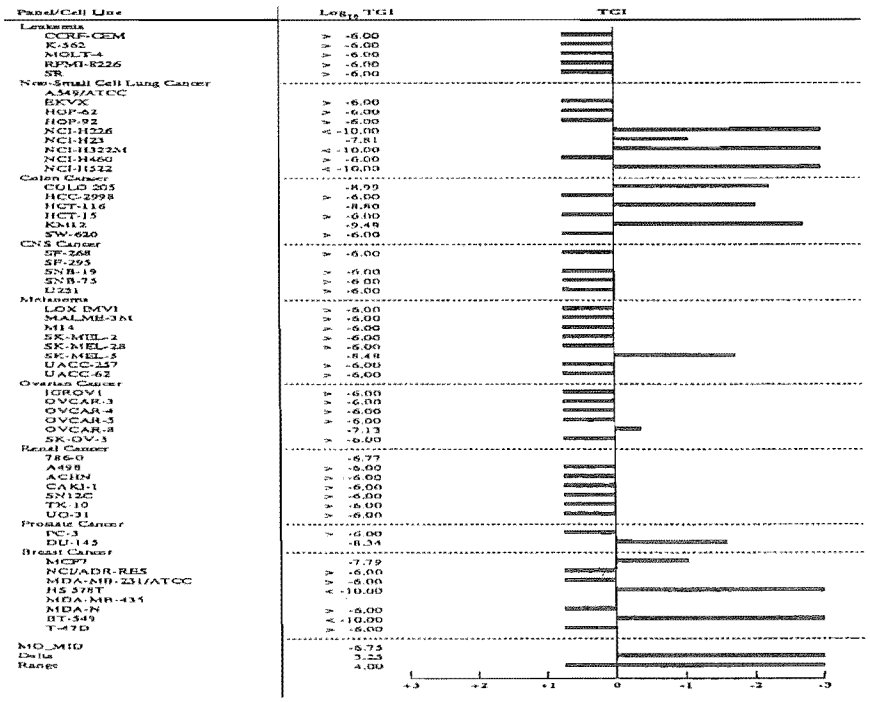
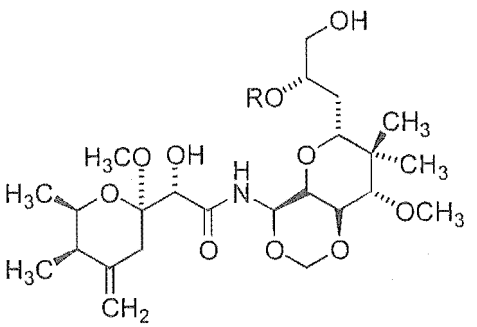


Figure 1.2.3 Mean Graph Profile for Norhomohalichondrin B (**4.17**)

Compounds that exhibit differential cytotoxicity for specific cell lines or panels in the screen are of most interest as they may represent a new class of disease-specific compounds and/or a new mechanism of action. The distinctive mean-graph "fingerprint" can also give an indication of the mechanism of action of a particular class of compounds. The NCI developed a pattern recognition algorithm, COMPARE, that enables the evaluation of patterns of cytotoxicity against the 60 cell lines obtained with a test agent to be compared to those obtained with all other agents in their data base.[43] A COMPARE correlation coefficient indicates the degree of similarity between the patterns of two agents. Seed compounds with known mechanisms of action can be used to predict the mechanism of action of test agents. For example, halichondrin B (**1.8**) correlated most closely to known antimitotic agents (see Section 1.2.4.2 for further discussion).

1.2.3.3 Bioassay-Guided Isolations

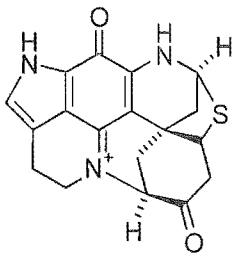
Bioassay-guided isolation of the active components from the Otago Harbour sponge *Mycale* sp. yielded mycalamide A (**1.18**) and mycalamide B (**1.19**).[44,45] The mycalamides exhibited antiviral activity but proved too cytotoxic to be effective ant-viral agents. The mycalamides were also rejected as antitumour agents after *in vivo* trials highlighted problems with toxicity.



R=H mycalamide A (**1.18**)

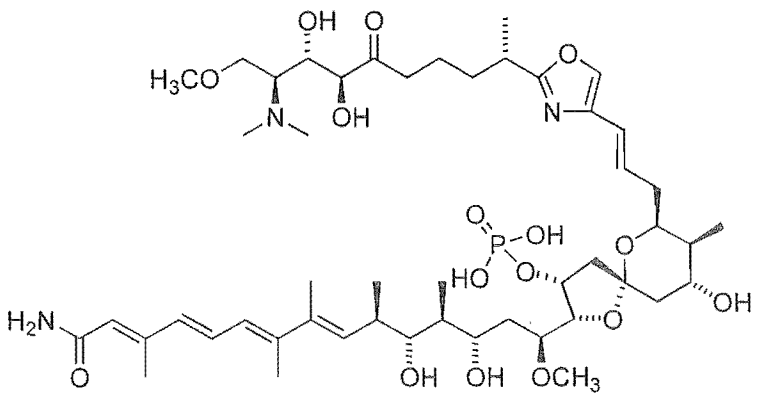
R=CH_3 mycalamide B (**1.19**)

The discorhabdins, a series of iminoquinones, were isolated from New Zealand species of the highly pigmented sponge *Latrunculia*.[46,47,48] Several discorhabdins exhibited promising *in vitro* activity against the P388 cell line (in the order of 20 ng/mL), but only discorhabdin D (**1.20**) exhibited (modest) *in vivo* activity (T/C 132% at 20 mg/kg).[48]

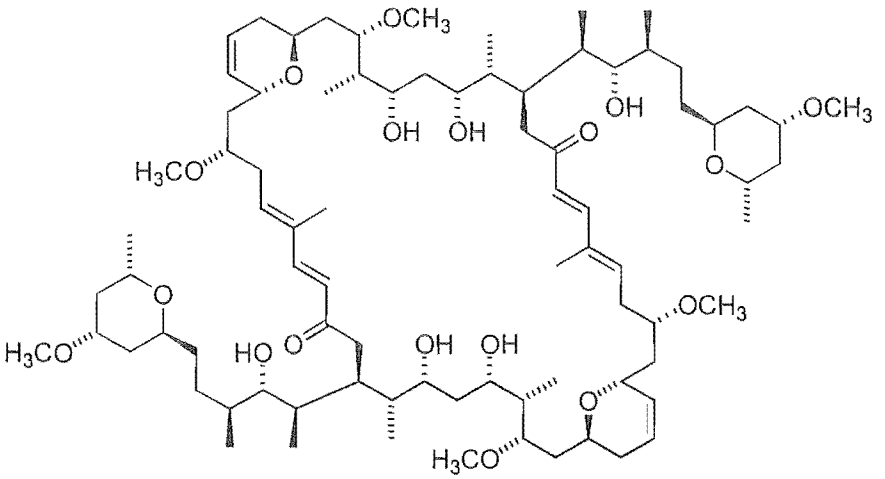


discorhabdin D (**1.20**)

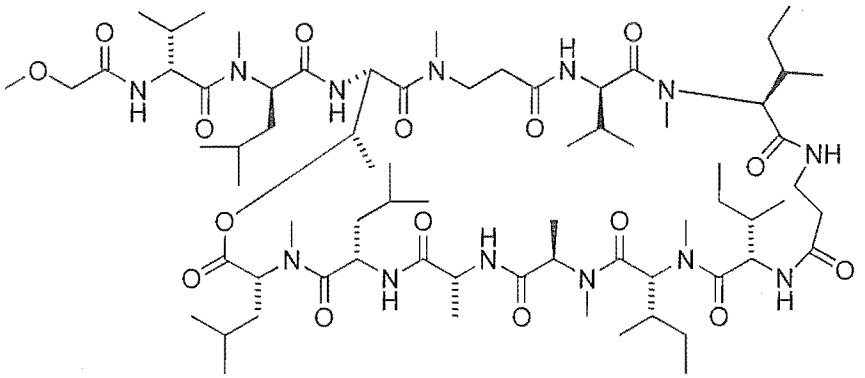
Bioassay-guided purification of the active components from an extract of *Lamellomorpha strongylata* yielded calyculinamides A (**1.21**) and B, swinholide H (**1.22**), a mixture of the previously reported calyculins A, B, E, and F,[49,50] and a mixture of theonellapeptolides.[51] The theonellapeptolides were subsequently characterised by Shangxiao Li.[52] Swinholide H and the calyculinamides were shown to have differential antitumour activity against the NCI's 60 cell line primary screen. Theonellapeptolide IIIe (**1.23**) exhibited modest *in vitro* cytotoxicity (7.4 μg/mL) against the P388 cell line.[52]



calyculinamide A (**1.21**)



swinholide H (**1.22**)



theonellapeptolide IIIe (**1.23**)

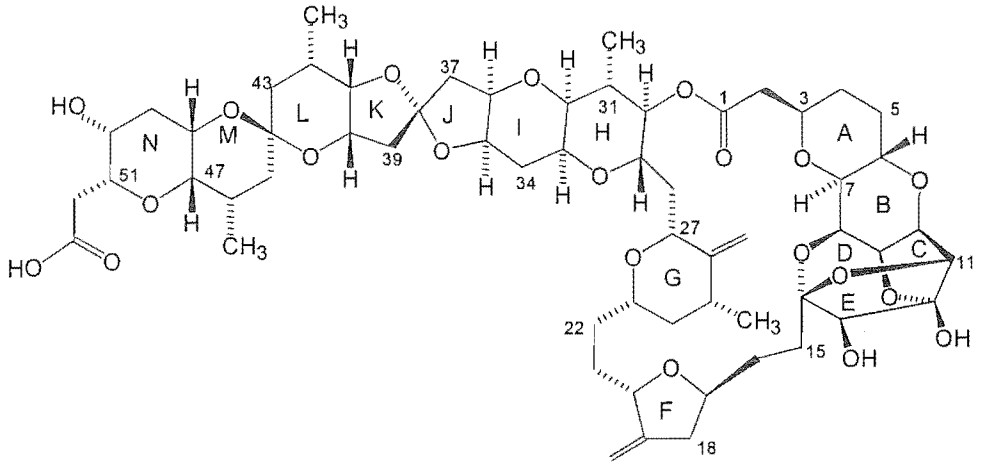
Bioactivity-guided fractionation of an extract of the Kaikoura sponge *Lissodendoryx* sp. yielded several members of the halichondrin family including halichondrin B (**1.8**). These potent antitumour polyether macrolides are discussed in more detail in Section 1.2.4.

1.2.4 The Halichondrins

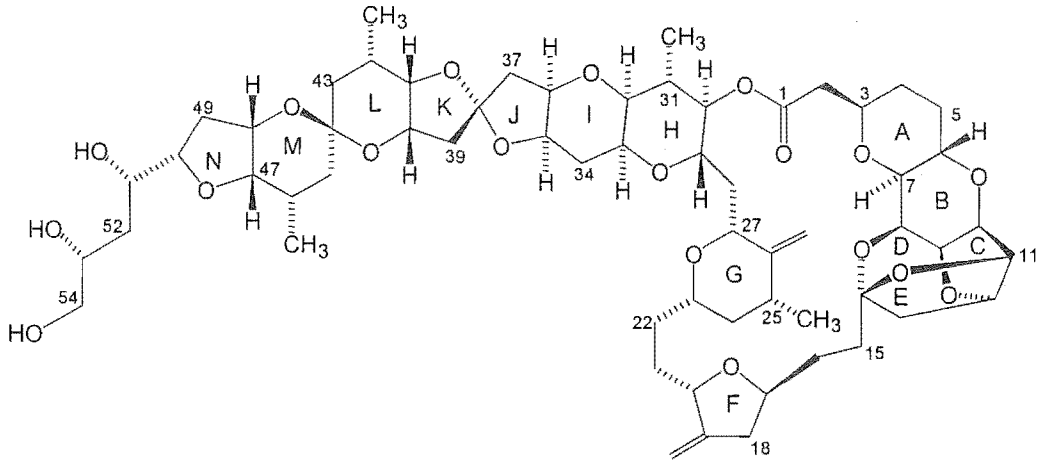
1.2.4.1 Discovery

Norhalichondrin A (**1.24**) was the first in a series of potent antitumour compounds reported by Uemura *et al* in 1985.[18] The isolation of a further seven halichondrins from the same Japanese sponge (*Halichondria okadai* Kadota) was subsequently reported in

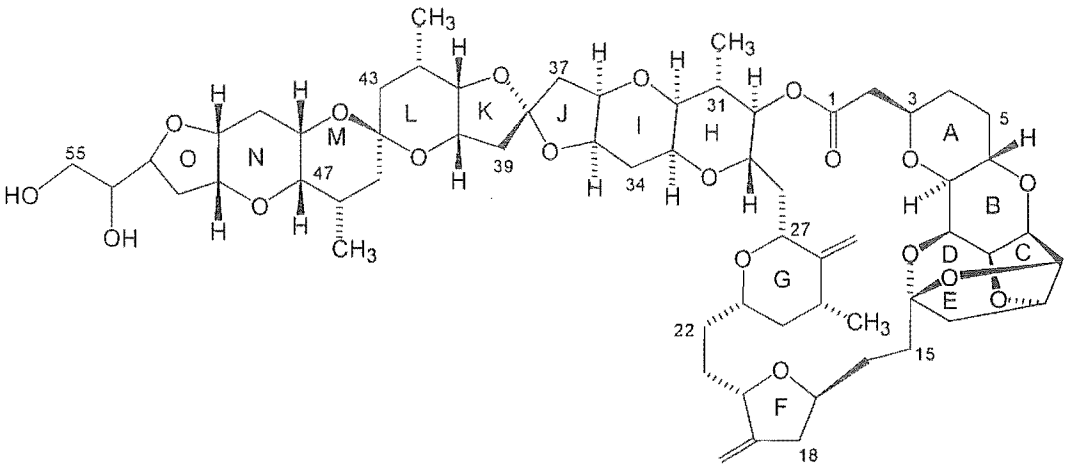
1986.[53] These halichondrins: norhalichondrin A (**1.24**), B and C, halichondrin B (**1.8**) and C, and homohalichondrin A, B (**1.25**) and C represent three classes of halichondrin. The A, B and C families are distinguished by the degree of oxidation at C12 and C13 (Figure 1.2.4). Within each family variation occurs beyond the C45 position.



norhalichondrin A (**1.24**)



halichondrin B (**1.8**)



homohalichondrin B (**1.25**)

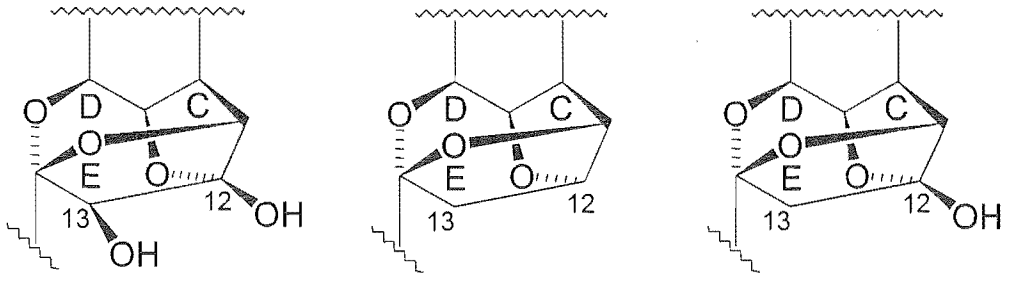


Figure 1.2.4 Halichondrin A, B and C Families

The structures are characterised by a novel 2,6,9-trioxatricyclo[3.3.2.$0^{3,7}$]decane system (rings C-E), a 22-membered lactone ring (C1-C30), two exocyclic olefinic groups and several pyranose and furanose rings.

Comparison of the cytotoxicity of all of the halichondrins isolated against the *in vitro* B-16 melanoma cell-line (Table 1.2.1) showed that halichondrin B (**1.8**) was the most cytotoxic. Halichondrins with B-type C-E rings and homo- or hali-type terminal moieties gave the best results.

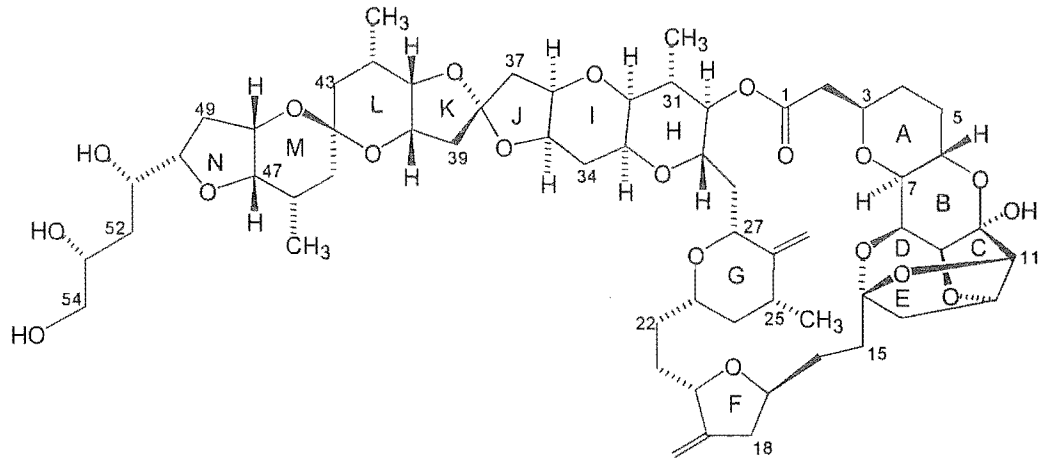
Table 1.2.1 Cytotoxicity against B-16 Melanoma cells (ng/mL)

Compound	IC_{50} (ng/mL)
Homohalichondrin A	0.26
Norhalichondrin A (1.24)	5.2
Halichondrin B (1.8)	0.093
Homohalichondrin B (1.25)	0.1
Norhalichondrin B	NR[a]
Halichondrin C	0.35
Homohalichondrin C	NR[a]
Norhalichondrin C	NR[a]

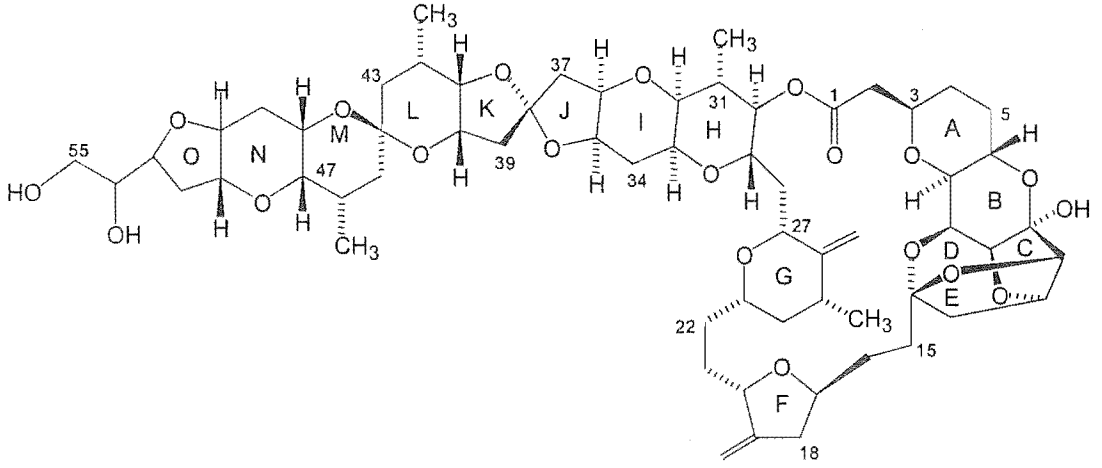
[a] Data not reported (NR) but result inferior to that for halichondrin B.[53]

The *in vivo* antitumour activity of halichondrin B against B-16 melanoma, P388 leukemia and L-1210 leukemia cell-lines was also determined. P388 leukemia infected mice treated with halichondrin B (**1.8**) experienced a 300% increase in median survival time relative to the control group.

The isolation of halichondrin B (**1.8**) and homohalichondrin B (**1.25**) from an unrelated sponge *Axinella* sp., collected in Palau, was reported in 1991.[54] Two new halichondrin compounds, halistatin 1 (**1.26**)[55] and halistatin 2 (**1.27**),[56] were reported by Pettit in 1993. Halistatin 1 (**1.26**) is 10α-hydroxyhalichondrin B and halistatin 2 (**1.27**) is 10α-hydroxyhomohalichondrin B. Halistatin 1 (**1.26**) was isolated from an east Indian Ocean sponge *Phakellia carteri*. Halichondrin B (**1.8**) and homohalichondrin B (**1.25**) were also isolated from this sponge. These three halichondrins were subsequently isolated from an extract of the Western Indian Ocean sponge *Axinella* cf. *carteri* Dendy together with halistatin 2 (**1.27**). Halistatin 1, halistatin 2 and halichondrin B were tested in the NCI's human tumour primary *in vitro* screen. The overall mean GI_{50} values were 7.1×10^{-10} M, 6.8×10^{-10} M and 2.3×10^{-10} M respectively.[56]



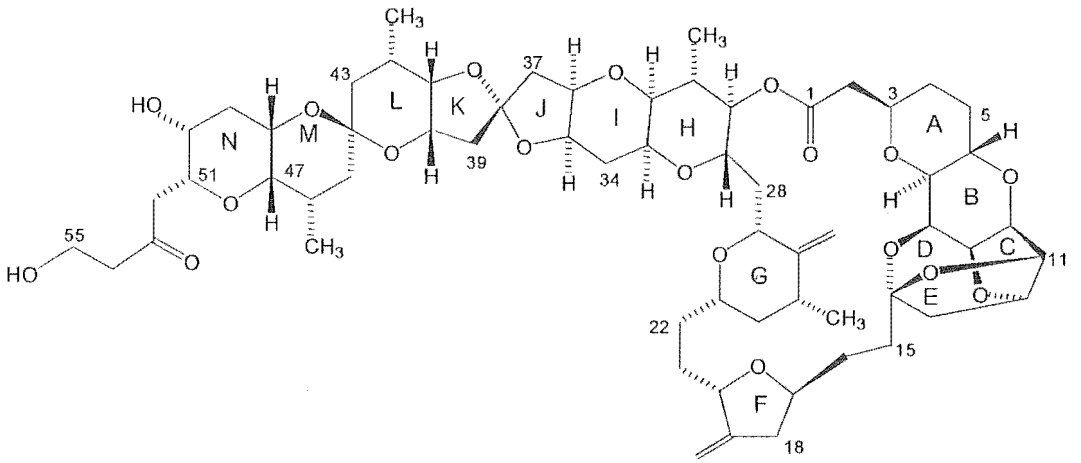
halistatin 1 (**1.26**)



halistatin 2 (**1.27**)

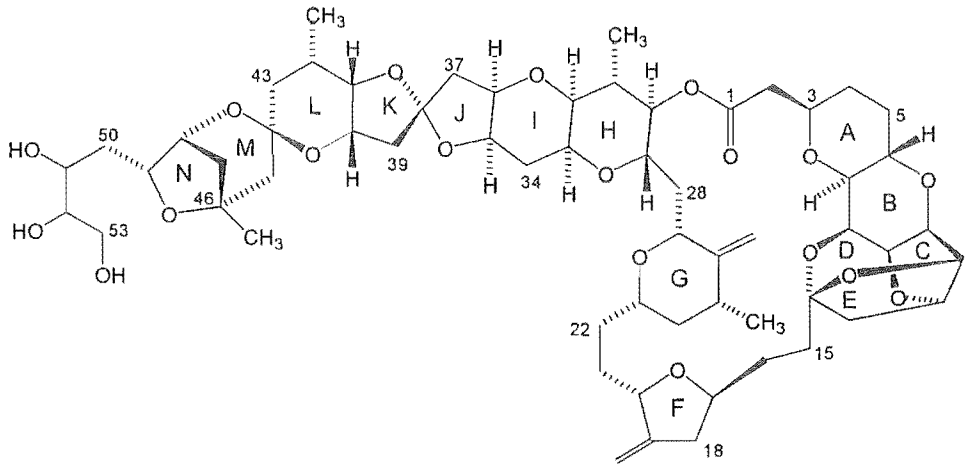
In 1987 halichondrin-like compounds were identified in two unrelated New Zealand species of sponge by Rob Lake. Trace quantities of halichondrins were detected in an extract of *Raspalia agminata* (order Axinellida, family Raspailiidae), a black shallow-water sponge from the Leigh area of the North Island.[57] A slimy yellow sponge obtained by benthic dredging (*ca* 100 m) off the Kaikoura coast was identified as one or more new species of the genus *Lissodendoryx* Topsent (class Demospongiae, order Poecilosclerida, family Myxillidae).[58] The Kaikoura sponge is higher yielding (~ 1 mg total halichondrins/kg wet weight of sponge) than any of the other halichondrin-producing sponges.[53,54,55]

A novel halichondrin-type compound was isolated by bioassay-directed fractionation of the first (small) extract of *Lissodendoryx* sp. Full characterisation of this compound was not achieved.[57] Two other halichondrins isolated from the extract were identified as halichondrin B (**1.8**) and homohalichondrin B (**1.25**). A subsequent extraction of *Lissodendoryx* sp. by Marc Litaudon yielded 4.5 mg of the unidentified halichondrin. This enabled the characterisation of the novel halichondrin using 2D NMR and MS techniques. Isohomohalichondrin (**1.28**)[59] was so named because it is isobaric to homohalichondrin B (**1.25**). The difference between the two compounds exists beyond C48, where isohomohalichondrin B (**1.28**) has the ring opened form of the terminal furanose unit of **1.25**.

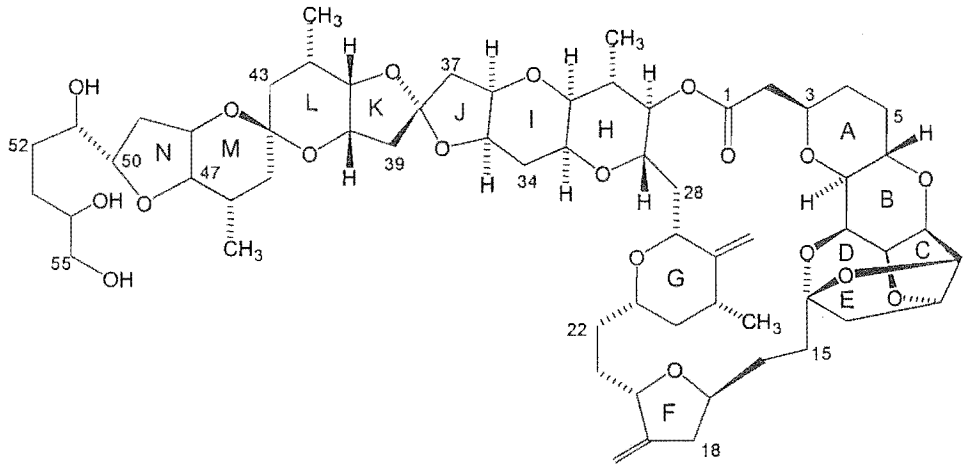


isohomohalichondrin B (**1.28**)

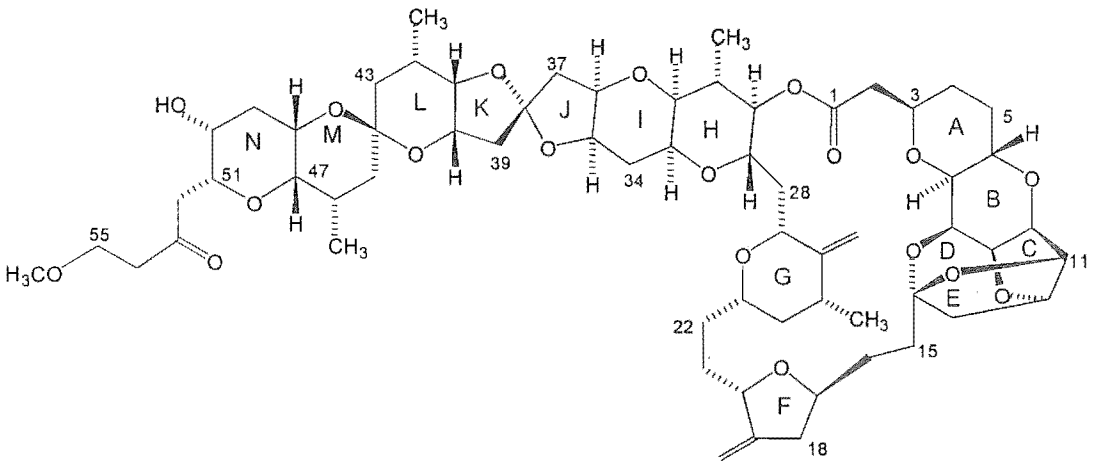
A bulk extraction of *ca* 200 kg of *Lissodendoryx* sp. yielded halichondrin B (43 mg), homohalichondrin B (45 mg) and isohomohalichondrin B (57 mg) together with norhalichondrin B (0.6 mg) and several novel minor halichondrin compounds.[60] Four of these minor compounds have been isolated and their structures established: neonorhalichondrin B (**1.29**, 0.9 mg), neohomohalichondrin B (**1.30**, 7 mg), 55-methoxyisohomohalichondrin B (**1.31**, 0.7 mg) and 53-methoxyneoisohomohalichondrin B (**1.32**, 15 mg).



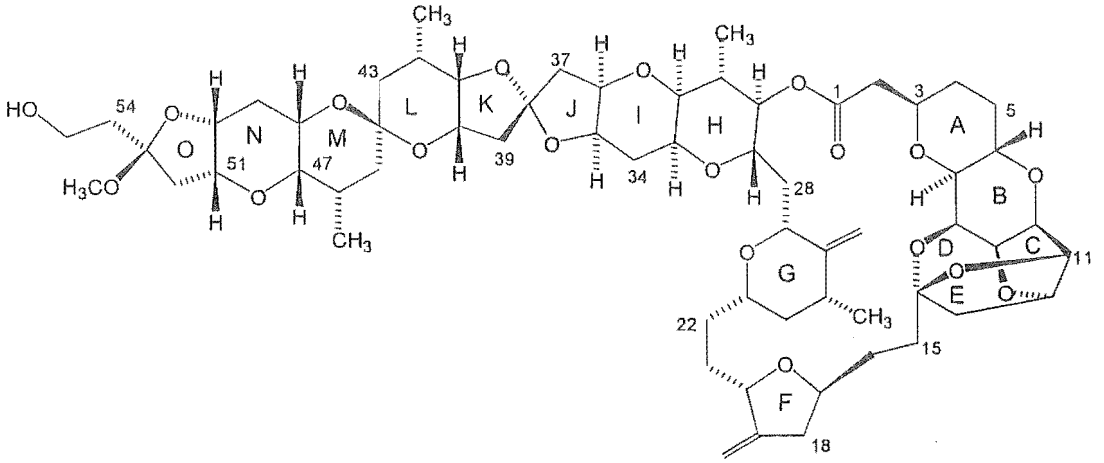
neonorhalichondrin B (**1.29**)



neohomohalichondrin B (**1.30**)



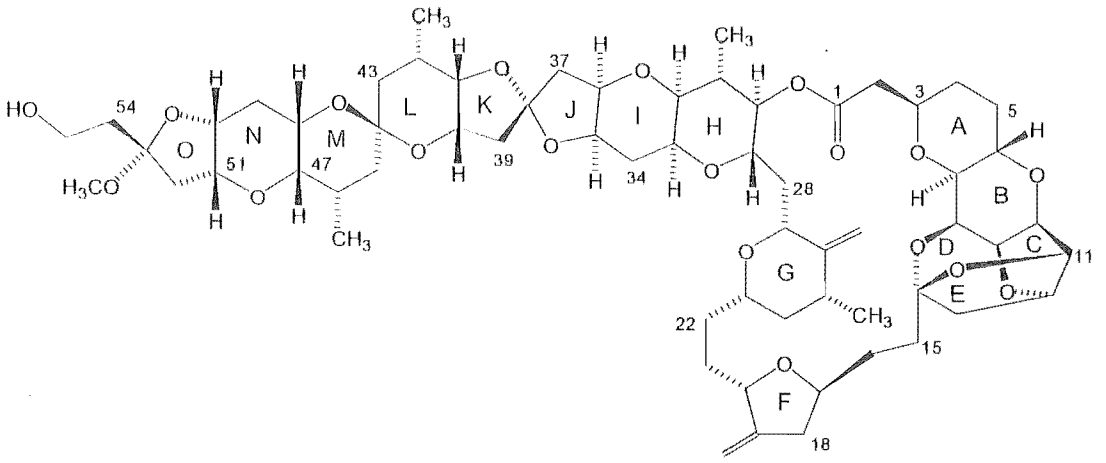
55-methoxyisohomohalichondrin B (**1.31**)



53-methoxyneoisohomohalichondrin B (**1.32**)

During the investigations on 53-methoxyneoisohomohalichondrin B (**1.32**) it was noted that the ^{1}H NMR spectrum of a sample kept in $CDCl_3$ without added CD_5N slowly reverted to a spectrum characteristic of isohomohalichondrin B (**1.28**).[60] In addition, a sample of **1.28** in CD_3OD was seen to revert over a period of time to **1.32**. This would indicate that **1.32** is likely to be an artefact of the isolation process, where methanol used for both extraction and chromatography caused the conversion of **1.28** to **1.32**.

A compound isobaric with **1.32** was also isolated (**1.33**, 1.5 mg) from the *Lissodendoryx* sp. extract. Like **1.32**, this compound was observed to revert to **1.28** in $CDCl_3$ solution (without added CD_5N). The instability of **1.33** precluded the collection of a full set of spectroscopic data. The ^{1}H NMR spectrum of **1.33** was identical to that of **1.32** with the exception of resonances for H51 and 53-OCH_3, suggesting the structure to be 53-*epi*-methoxyneoisohomohalichondrin B.[60]



53-*epi*-methoxyneoisohomohalichondrin B (**1.33**)

The halichondrins isolated from *Lissodendoryx* sp. were tested for antitumour activity in the Marine Chemistry Group's in-house P388 *in vitro* murine leukemia assay, and against the NCI's 60 cell-line *in vitro* primary screen. The results are shown in Table 1.2.2.

Table 1.2.2 *In Vitro* Cytotoxicities of Halichondrins from *Lissodendoryx* sp.

Compound	**P388 IC_{50} (ng/mL)**	**NCI GI_{50} ($\times 10^{-10}$ M)**
halichondrin B (1.8)	0.78	1.38
homohalichondrin B (1.25)	0.22	3.16
isohomohalichondrin B (1.28)	0.18	1.15
norhalichondrin B	not recorded	29.50
neohomohalichondrin B (1.30)	0.8	3.39
methoxyneoisohomohalichondrin B (1.32)	0.1	1.23
methoxyisohomohalichondrin B (1.31)	10.0	2.00
neonorhalichondrin B (1.29)	0.4	12.30

Methoxyneoisohomohalichondrin B (**1.32**), isohomohalichondrin B (**1.28**) and neohomohalichondrin B (**1.30**) have comparable cytotoxicities to those of halichondrin

B (**1.8**) and homohalichondrin B (**1.25**) in both assays. The NCI screen is relatively insensitive to neonorhalichondrin B (**1.29**) compared to the P388 cell-line. The opposite is true of methoxyisohomohalichondrin B (**1.31**), which exhibits comparable cytotoxicity to that of methoxyneoisohomohalichondrin B (**1.32**) in the NCI screen yet is a hundred-fold less active against the P388 cell line.

Halichondrin B has since undergone further biological testing at the NCI. It is highly potent and differentially active in the sixty cell line *in vitro* cancer screen run by the Development Therapeutics Program (DTP). Halichondrin B has been demonstrated to decrease tumour growth rates in several human tumour xenograft models, including LOX IM IV (melanoma), NCI-H522 (lung), OVCAR-3 (ovarian) and MDA-MB-435 (breast),[61] and currently has Decision Network IIA status with the NCI.[62] The progression to Decision Network IIB status is unlikely to be made until arrangements for the purchase or supply of a bulk (10 g) quantity of the compound can be made. The implications of this are discussed in more detail in Section 1.2.4.3.

1.2.4.2 Mode of Action

An evaluation of the pattern of differential activity (mean-graph profile) of halichondrin B (**1.8**) against the NCI 60 human tumour cell line panel using the COMPARE[43] algorithm found that it most closely resembled those of the tubulin binding agents[63] such as vincristine and taxol. This suggested that halichondrin B (**1.8**) was an antimitotic agent.

Microtubules are tubulin polymers involved in many cellular functions including formation of the mitotic spindle required for chromosome segregation during cell division. Tubulin exists as a dimer consisting of α and β subunits. Protofilaments are formed from the head to tail arrangement of heterodimers of α and β tubulin, in the presence of guanosine triphosphate (GTP). These protofilaments group together to form a protein sheet that curls to form the microtubule.[64] A third type of tubulin protein, γ-

tubulin is essential for microtubule growth *in vivo*. Two molecules of GTP are bound to the heterodimers, one of which is freely exchangeable with unbound GTP. It is widely thought that this exchangeable GTP molecule is involved in the regulation of tubulin function.[64] Microtubule destabilising agents tend to bind to the free dimer, preventing polymerisation.

A significant characteristic of a cancer cell is that it divides more frequently than a normal healthy cell. Since microtubules are involved in cell proliferation (mitosis), microtubule poisons are a potent tool in the fight against cancer. It should be noted that antimitotic agents interrupt microtubule assembly in all rapidly dividing cells. This indiscriminate action, typical of the current breed of cancer chemotherapeutics, is responsible for the unpleasant side effects associated with cancer treatment.

Drugs which bind to tubulin can be subdivided into separate classes depending on the effect that drug has on the binding of five well characterised tubulin binding agents to tubulin. The drugs are categorised depending on whether they prevent the binding of colchicine, the binding of the vinca alkaloids (vincristine and vinblastine) or the binding of rhizoxin and maytansine to tubulin.[64] Some drugs do not interfere with the binding of these agents, having affinity for a separate distinct region of tubulin.

The vinca alkaloids vinblastine and vincristine, isolated from the periwinkle *Catharanthus roseous*, have been widely used as clinical agents in the treatment of leukemias, lymphomas and some solid tumours.[64] Both these agents induce the destabilisation of polymerised tubulin by binding to a site recently localised on β-tubulin.[64] The "vinca site" represents the region of tubulin where the vinca alkaloids and their competitive inhibitors (*eg* maytansine) bind. The peptide antimitotics dolastatin 10 and phomopsin A are non-competitive inhibitors of the vinca alkaloid binding to tubulin. This is interpreted as binding in a region physically near to, but distinct from, the vinca site. This site is termed the "vinca domain".

Investigations by Bai *et al*[63] noted the accumulation of cells arrested in mitosis at cytotoxic concentrations of either halichondrin B (**1.8**) or homohalichondrin B (**1.25**) for L1210 murine leukemia cells *in vitro*. These compounds were found to inhibit the polymerisation of purified tubulin and microtubule assembly dependent on microtubule associated proteins. Halichondrin B was found to be a relatively weak inhibitor of tubulin polymerisation, despite its potent cytotoxicity. Bai[63] speculated that this was a reflection of either the highly efficient uptake of the drug or its intracellular metabolism to more potent compounds. Halichondrin B did not interfere with colchicine binding to tubulin, but was found to be a non-competitive inhibitor of the vinca alkaloid vinblastine. This suggested that halichondrin B was binding in the vinca domain of tubulin. Vinca domain drugs inhibit tubulin-dependent GTP hydrolysis and also inhibit GTP/GDP exchange at the exchangeable nucleotide site of tubulin. Halichondrin B was found to inhibit net GTP hydrolysis and nucleotide exchange on tubulin. These observations verified the earlier COMPARE differential cytotoxicity analysis that the mode of action for halichondrin B was as an antimitotic agent.

Isohomohalichondrin B (**1.28**) has also been shown to break up the microtubule network by direct interaction with tubulin, resulting in the decreased binding of GTP to the protein.[65] Luduena *et al*[66] suggest that halichondrin B (**1.8**) has a unique conformational effect on the tubulin molecule.

1.2.4.3 Supply Issues

The further preclinical development of halichondrin B (**1.8**) requires gram quantities of halichondrin B. The clinical use of halichondrin B would require *ca* 5 kg/year. This would necessitate the annual production and extraction of at least 5000 tonnes of *Lissodendoryx* sp.[68] The three main supply options are the harvesting of native sponge, synthesis of the target compound and sponge aquaculture.

Native Sponge Harvest

The halichondrin material provided for testing to date has originated from the extraction of native sponge. As mentioned previously (Section 1.2.4.1), *Lissodendoryx* sp. is high yielding relative to other halichondrin-containing sponges. *Lissodendoryx* sp. is, however, a rare deep water species found exclusively off the Kaikoura peninsula.[67] An extensive environmental survey, conducted using an ROV and a benthic camera, established that the "sponge field" was only ~ 5 km^2, with the mean biomass and abundance of sponge estimated to be 69 ± 21 g/m^2 with 1.1 ± 0.1 individuals/m^2 over the sponge field.[68] This survey gave a total estimate of *Lissodendoryx* sponge of 289 ± 90 tonnes.[67,69] This result is significant, in that it means that the commercial supply of halichondrins from native *Lissodendoryx* sponge is not viable.

Synthesis

Halichondrin B (**1.8**) has received much attention due to its potent *in vivo* antitumour activity. The scarcity of the material from natural sources has encouraged considerable synthetic effort in this area.[70] The total synthesis of halichondrin B (**1.8**) and norhalichondrin B was achieved by Kishi and coworkers in 1991.[71]

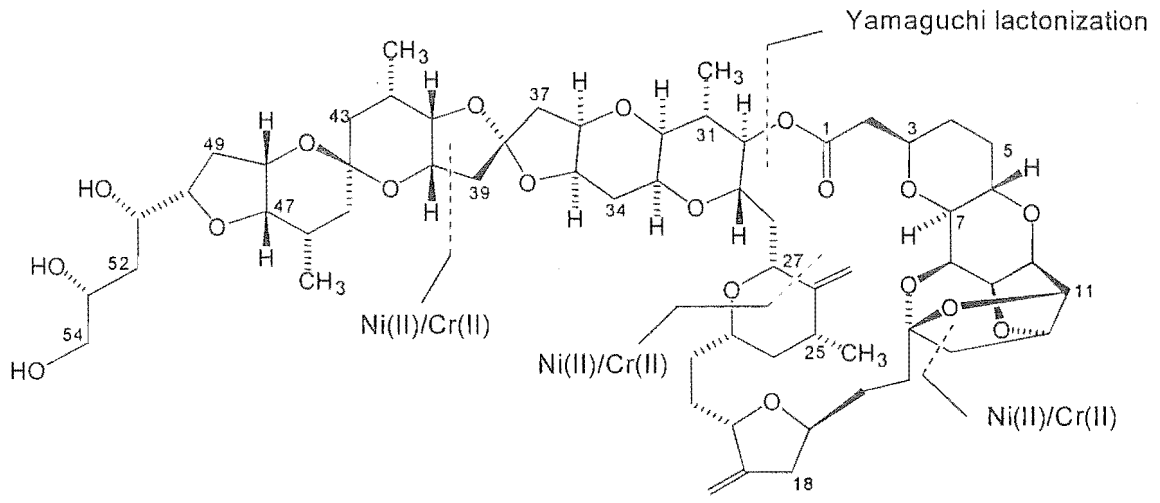


Figure 1.2.5 Strategic Couplings used in Kishi's Total Synthesis of Halichondrin B

The strategically important couplings in Kishi's total synthesis of halichondrin B (**1.8**) are illustrated in Figure 1.2.5. The construction of the C13-C14, C38-C39 and C26-C27 bonds was achieved by Nozaki-Hiyama-Kishi Ni(II)/Cr(II)-mediated coupling. This reaction was utilised several times in the synthesis. Yamaguchi lactonization was used to close the macrocycle. Simple sugars were used to provide ~ 50% of the stereogenic centres in halichondrin B.

The major fragments utilised in Kishi's synthesis are shown in Figure 1.2.6. Fragment A was synthesised in eighteen steps from an *L*-ascorbic acid-derived lactone (fragment D). The synthesis of fragment B required fifty four steps from *D*-galactose glycal. Fragment C was obtained in sixteen steps from *L*-mannonic-γ-lactone. Coupling of these fragments together *via* two Nozaki-Hiyama-Kishi (Ni(II)/Cr(II)) couplings and macrolactonisation followed by further manipulations gave halichondrin B (**1.8**) in *ca* 120 steps. A twelve step synthesis of fragment C was reported in 1996.[72]

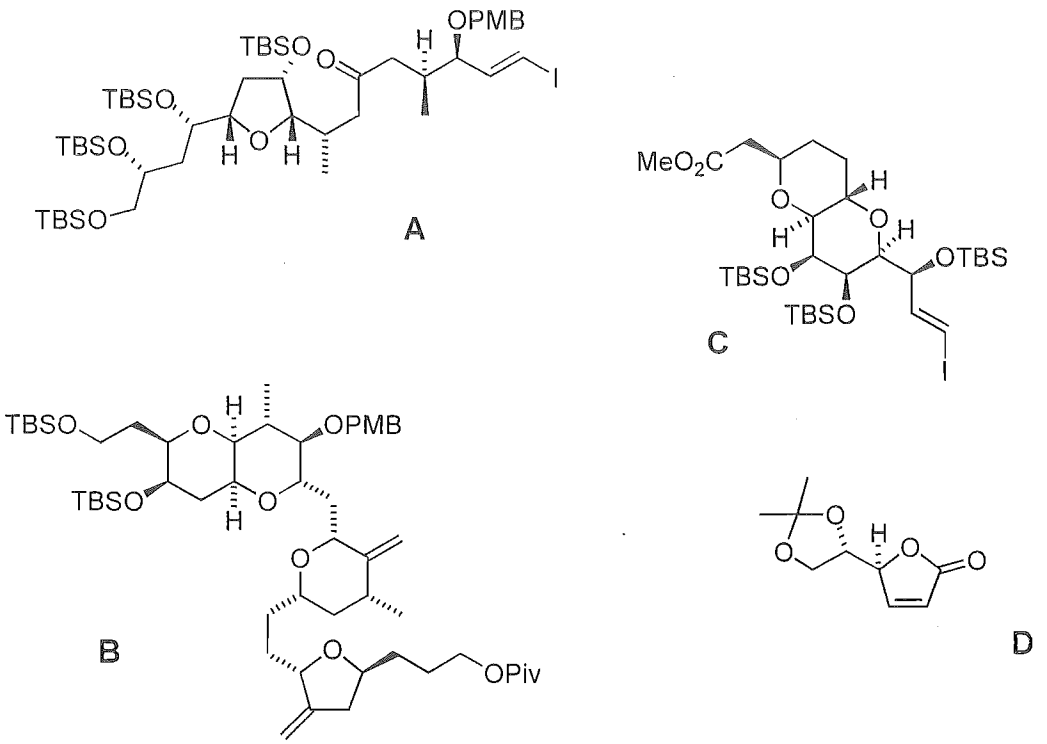
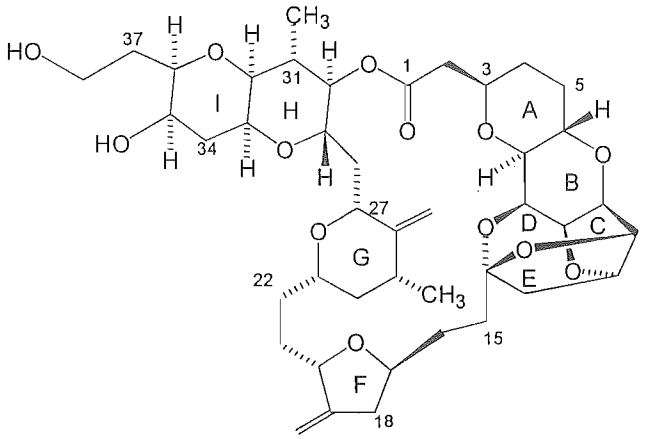


Figure 1.2.6 Major Fragments in the Kishi Synthesis of Halichondrin B (**1.8**).

It was recently reported that the right-half diol (**1.34**) exhibits the same activity pattern as the parent halichondrin B (**1.8**). The cytotoxicity of this truncated halichondrin was also within one order of magnitude of that exhibited by **1.8**.[73] This discovery has concentrated synthetic efforts on the C1-C38 segment of halichondrin B. A new approach to C14-C38 segment was reported by Stamos *et al* in 1997.[73] Although this strategy actually contains additional steps, it is an improvement in terms of a practical scalable synthesis.



right-half diol (**1.34**)

Significant contributions to the synthesis of halichondrin B have also been made by the groups of Salomon, Horita and Yonemitsu, and Burke.[70] In some cases the synthesis of a particular halichondrin fragment is considerably more expeditious than in Kishi's total synthesis, but to date none of these fragments have been utilised in a total synthesis. Consequently it is difficult to comment on the viability of these approaches as a source of halichondrins.

The commercial supply of halichondrin B (**1.8**) by total synthesis is clearly very unlikely. However, the synthesis of the right-half diol (**1.34**) may offer the possibility of a commercially viable synthesis.

Aquaculture

The farming of marine organisms as a source of pharmaceuticals is still in its infancy. One success story is the aquaculture of *Bugula neritina*, a colonizing bryozoan, for the large scale production of the antitumour agent bryostatin 1 (**1.6**). It has been established that *B. neritina* aquacultured by CalBioMarine Technologies off the southern Californian coast produces bryostatin 1 in comparable yield to the wild population.[7]

The aquaculture of *Lissodendoryx* sp. is currently being trialed by the New Zealand National Institute of Water and Atmospheric Research (NIWA). In a preliminary study explants of the sponge were relocated from the deep water off Kaikoura (*ca* 100 m) to a more accessible shallow water location in Wellington Harbour. These explants had at least doubled in size over four months at this site, and in some cases growth rates of up to 500% were recorded.[74] It was also noted that small samples that had broken off had recruited neighbouring sites.[74] Further experiments were undertaken to establish optimal explant conditions and culture localities. It was quickly apparent that summer explants were not successful, with greater than 95% mortality.[69] In contrast winter explants were generally successful, especially at greater depths, with impressive growth rates through to December, when a decline was observed as bryozoan overgrowth and

pathogen attack became excessive.[69] A critical temperature of 18°C was identified, above which the sponges will not survive.[69]

The aquacultured sponges are grown in a significantly different environment to their native habitat. It is therefore important to determine if the organism still produces the secondary metabolites of interest. Extracts of *Lissodendoryx* sp. grown by aquaculture at several different sites were investigated as part of this thesis (Chapter 3).

Future Options

Genome transfer to a vector appropriate for fermentation requires the identification of the producing organism. This is not clear cut in the case of the halichondrins, with some thought that a symbiont could be responsible for their production. However, preliminary cell separation studies on *Lissodendoryx* sp. indicate that the halichondrins are associated with the sponge cells.[75] It is hoped a cell separation study of *Lissodendoryx* sp. currently being undertaken in the Marine Chemistry Group will confirm this result.

Sponge cell culture is also being investigated as a possible source of halichondrins. Attempts to develop long term, continuous cell lines from marine sponges are still very much in their preliminary stages. Indeed, it has recently been suggested that even the so-called long term cultures from several marine sponges are likely to be artefactual. The "sponge cells" that have been maintained in culture for an extended period of time have been identified as thraustochytrids.[76]

1.3 Immunoassay Development

In addition to the supply problem, an impediment to further preclinical and clinical studies of halichondrin B (**1.8**) is the lack of an adequate assay method. The remarkable

potency of halichondrin B (**1.8**) and the chemical complexity of the sponge extracts impose demanding requirements on the sensitivity and specificity of an assay.

The *in vivo* antitumour activity of halichondrin B (**1.8**) indicates that the dosage of halichondrin B will result in serum levels of 100 pg/mL or less.[62] Pharmacokinetic studies of halichondrin B and its metabolites will require a highly sensitive assay for determination of the parent drug and its major metabolites in serum and other body fluids. A highly sensitive assay is also required for the detection of contaminants in the drug preparation, such as other halichondrin congeners.

The halichondrins do not possess a chromophore that can be readily exploited for detection by conventional UV or visible spectroscopic methods. HPLC with refractive index detection or UV detection at 199 nm is the current detection method. This method requires several chromatographic steps prior to HPLC analysis, is time consuming, expensive, and not particularly accurate. The detection limit of HPLC analysis (> 0.01 mg/mL) does not meet the criteria of a suitable assay method for pharmacokinetic studies of halichondrin B (**1.8**).

Immunoassays offer rapid, specific and cost-effective means of identifying and quantitating trace amounts of biologically significant compounds such as proteins, hormones, peptides, drugs, pesticides and natural toxins in complex biological matrices.[77] Owing to the high antigen specificity of antibodies, crude biological extracts or fluids often require less purification prior to analysis.[78]

1.4 Polymer Therapeutics

1.4.1 Introduction

The treatment of solid tumours using conventional chemotherapeutics is often not possible because the toxic side effects of these antiproliferative drugs to surrounding

healthy tissue prevents administration of an efficacious dose. The polymer therapeutics approach of conjugating cytotoxic drugs to inactive polymers is providing a new class of anticancer agents that offer improved pharmacokinetic properties and enhanced selectivity for tumour tissue.

Ringsdorf first suggested the use of a water soluble polymer as a drug carrier incorporating a biodegradable linker to facilitate controlled release of the drug at the tumour site in 1975.[79] This area has been well studied since then, culminating in regulatory approval for two polymer-protein conjugates. A conjugate of styrene-co-maleic anhydride and the antitumour protein neocarzinostatin (SMANCS) has been approved in Japan for the treatment of liver cancer.[80] In the USA, FDA approval has been granted for a conjugate containing the polymer poly(ethyleneglycol), (PEG)-*L*-asparaginase, for the treatment of acute lymphocytic leukemia in children.[80]

1.4.2 Definition

A schematic of a polymer drug conjugate is shown in Figure 1.4.1. The major components of a polymer drug conjugate are the water soluble polymer, the biodegradable linker used to attach the drug, the drug itself, and an (optional) targeting residue. The specific requirements of the polymer, the linker and the targeting residue will be discussed in turn.

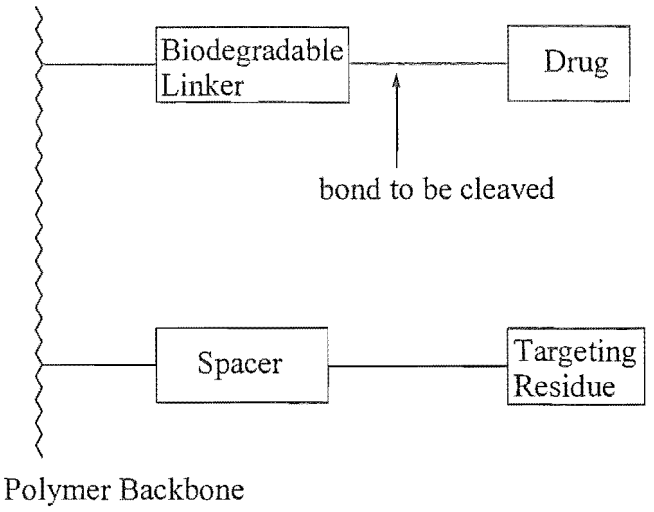


Figure 1.4.1 Features of a Polymer Drug Conjugate

1.4.2.1 Polymer

The polymer must be biocompatible. In other words it should not induce significant toxicity or immunogenicity. Unless the polymer is of sufficiently low molecular weight to permit excretion, the polymer must be biodegradable. Suitable functionality must be present within the polymer to permit conjugation to the bioactive agent *via* a linker whose chemistry does not impinge on the aforementioned requirement of biocompatability. It is important that the polymer have sufficient drug carrying capacity for the potency of the drug to be delivered. From a commercial point of view the polymer must be amenable to economically viable production on a commercial scale, and must yield a well characterised, stable formulation that can be manufactured reproducibly and can be conveniently administered to patients.[81]

1.4.2.2 Linker

The linker is required to be stable in circulation, enabling delivery of the drug specifically to the site of action. Once within the target cell, the linker must be biodegradable in some manner. Linkers can be designed to cleave at a particular pH. Alternatively, the linker can be tailored for specific enzymatic cleavage.

1.4.2.3 Targeting Residue

The incorporation of a targeting residue into the polymer drug conjugate offers the possibility of targeting specific cell types. Carbohydrates, hormones and antibodies (or fragments there from) are used to direct polymer conjugates to specific cell subsets.[82] Targeting moieties coupled to HPMA copolymer conjugates include galactosamine, melanocyte-stimulating hormone, transferrin, and various monoclonal and polyclonal antibodies.[83] An example of a polymer therapeutic incorporating galactosamine as a targeting residue is given in Section 1.4.5.

The use of targeting residues is hindered by the lack of specificity of many so-called specific receptors or antigens. Many of the receptors proposed as candidates for specific cell targeting have a broad cellular distribution. This can result in the removal of a large amount of the polymer therapeutic from circulation prior to accessing the tumour by normal tissue-mediated uptake.[83]

1.4.3 Cellular Uptake

The mechanism of cellular uptake for polymer-bound drug is contrasted with that of unbound drug in Figure 1.4.2.

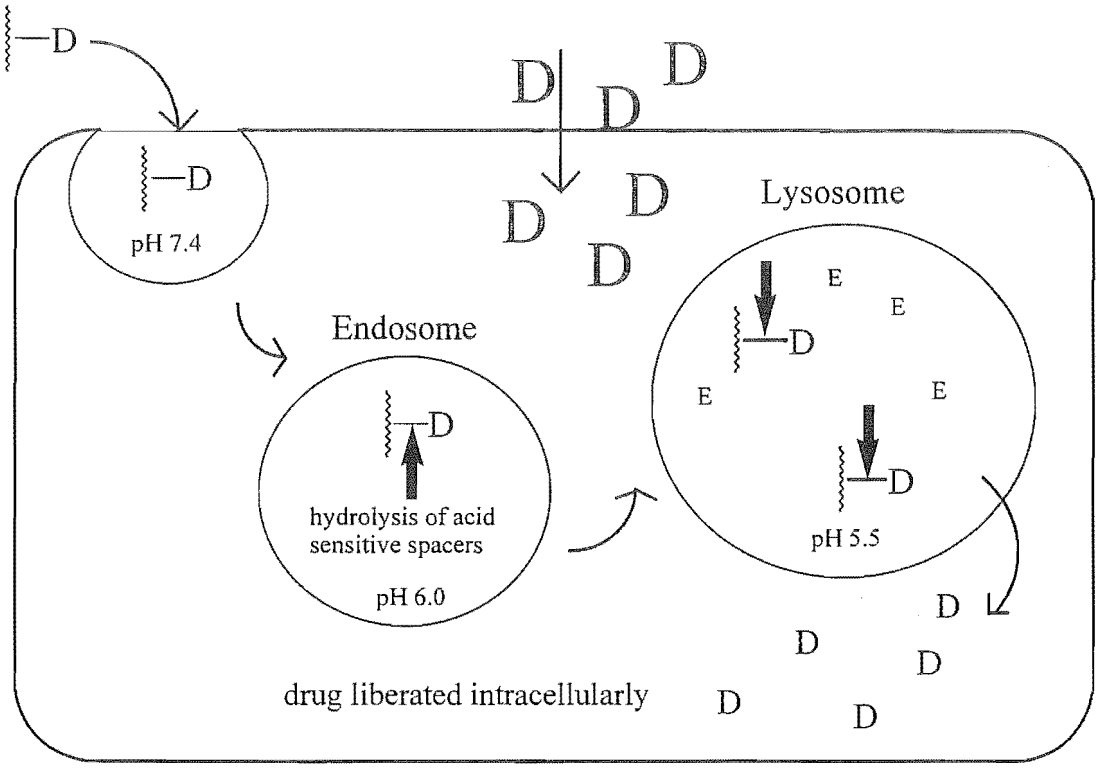


Figure 1.4.2 Comparison of Mechanism of Cellular Uptake of Free Drug and PDC

Macromolecules such as polymer drug conjugates cannot enter cells by diffusion in the manner of unbound low molecular weight drugs. The normal mechanism whereby a polymer passes through the cell membrane is by the process of endocytosis. This represents polymer conjugate engulfment by an infolding of the plasma membrane. The initiation of endocytosis is influenced by several factors such as molecular weight and charge effects.[79] Endocytosis delivers the polymer conjugate to the endosomal and subsequently the lysosomal compartments of the cell. This offers two distinct methods for drug liberation. The drug linker could be designed to take advantage of the drop in pH that occurs (see Figure 1.4.2). Alternatively, the linker could be tailored to specific cleavage by lysosomal enzymes.

1.4.4 EPR effect

The restriction of cellular uptake to endocytosis means that after intravenous administration the polymer drug circulates for longer in the blood stream. Consequently

the polymer drug accumulates more effectively in tumour tissue relative to conventional low molecular weight chemotherapeutics. This phenomenon, discovered by Hiroshi Maeda, is known as the enhanced permeability and retention (EPR) effect.[84] Figure 1.4.3 illustrates the two main factors giving rise to the EPR effect. The tumour vasculature often displays a discontinuous endothelium (*ie* is leaky) which allows macromolecular extravasation. The lack of effective lymphatic drainage within the tumour leads to accumulation of the polymer therapeutic at the desired site of action.

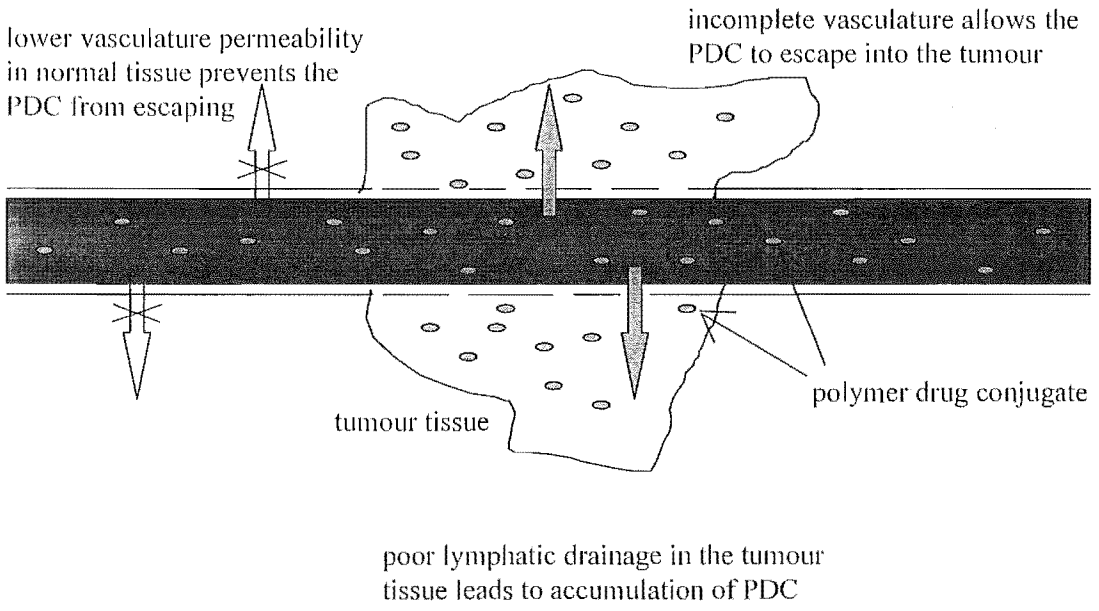


Figure 1.4.3 Enhanced Permeability and Retention (EPR) Effect

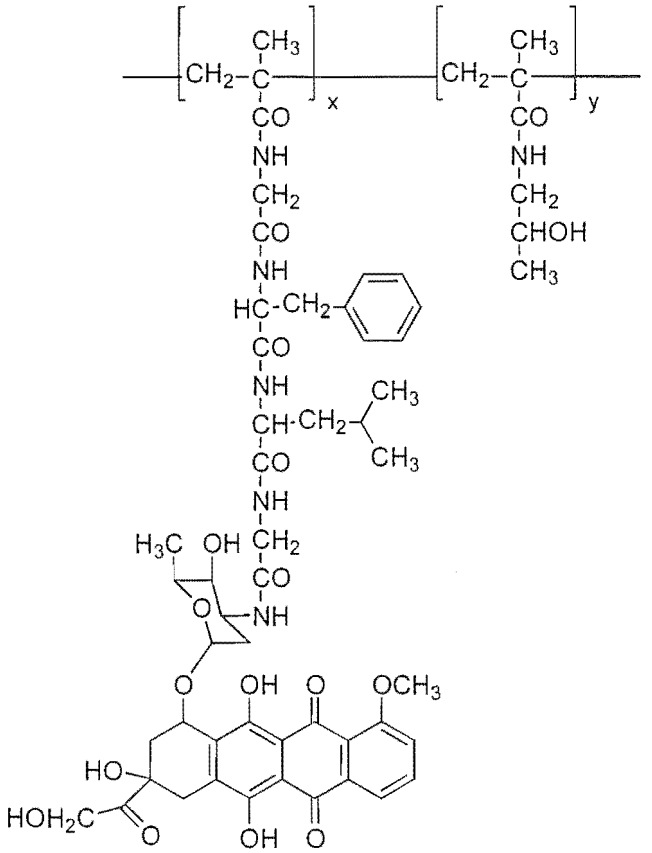
1.4.5 HPMA Copolymer Conjugates

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer conjugates have been studied extensively.[81] HPMA is a biocompatible polymer originally developed as a plasma expander.

Bioactive compounds (and targeting residues) containing an amino group can be conjugated to the reactive polymeric precursor in a simple aminolysis reaction. The antitumour agents are covalently bound to HPMA copolymers *via* peptidyl spacers

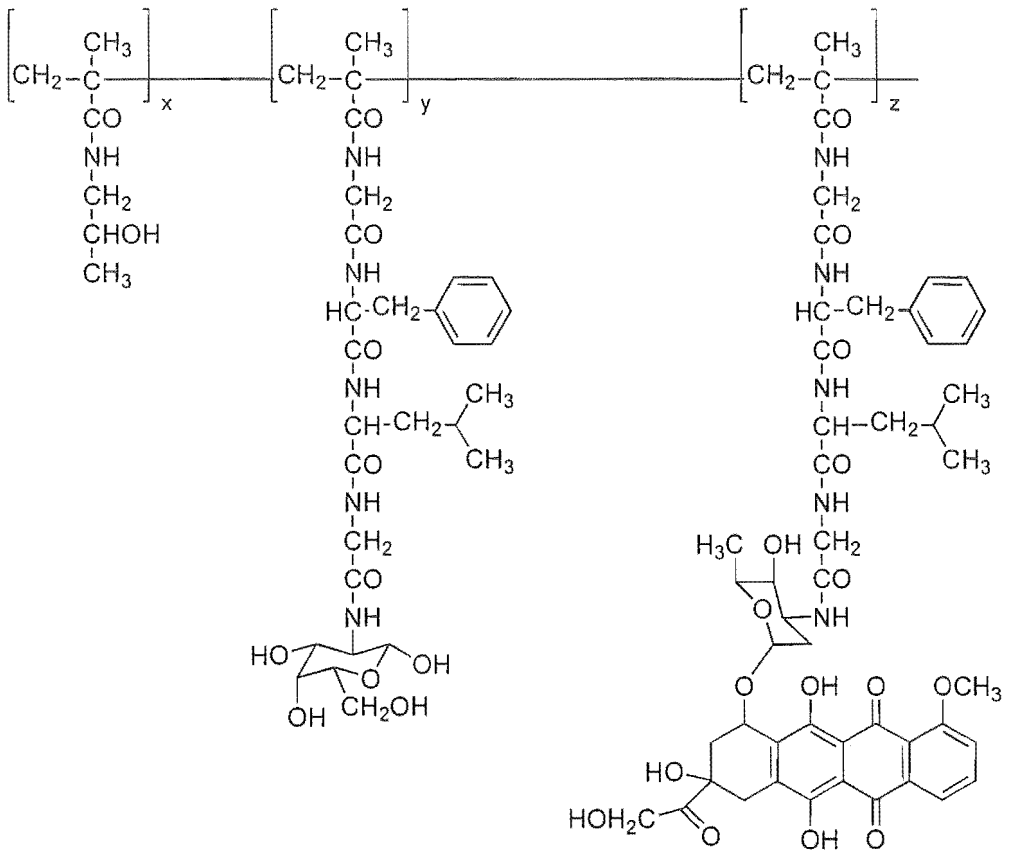
designed to limit drug release in plasma and serum, but be amenable to degradation by lysosomal proteases.[81]

Several compounds from this family are now in clinical study.[85] PK1 (**1.35**) is a simple HPMA copolymer conjugate containing the antitumour agent doxorubicin bound to the polymer backbone by a Gly-Phe-Leu-Gly peptidyl spacer.[85] Preclinical tests showed that PK1 was 5-10 times less toxic than free doxorubicin and displayed improved antitumour activity, particularly in solid tumour models.[85] Phase II trials are currently in progress.



PK1 (**1.35**)

An HPMA copolymer conjugate containing doxorubicin and employing the targeting residue galactose, PK2 (**1.36**) localises a large percentage of the doxorubicin dose in the liver.[85] It is hoped that this copolymer conjugate, currently in Phase I clinical testing, will be useful in the treatment of primary and secondary liver cancer.



PK2 (**1.36**)

1.5 Project Aims

The main focus of this thesis was the further development of the halichondrins with respect to their clinical viability. The importance of halichondrin B (**1.8**) as a potent *in vivo* antitumour agent is well established. Any modification to the halichondrins that improves their pharmacokinetic properties, increases selectivity, and reduces the required plasma concentrations has to be regarded as advantageous.

The development of a polymer therapeutic was viewed as a means of improving the chances of the future clinical development of halichondrin B (**1.8**). To this end, the development of a synthetic route to a halichondrin amine from homohalichondrin B (**1.25**) formed a major part of this thesis.

An efficient, selective and sensitive detection halichondrin assay would be required should halichondrin B (**1.8**) advance to clinical testing. The formation of haptens designed for antibody recognition of the macrocyclic portion of the halichondrins was undertaken. Presentation of the terminal end of the halichondrins giving rise to congener specific antibodies necessitated attachment of a linker somewhere in the lactone portion of the halichondrin skeleton. An important aspect of this thesis was the development of chemistry to facilitate the selective oxidation of one of the two exocyclic olefinic groups thus providing a functional group conducive to linker attachment.

Aquaculture seems to be a promising prospect for overcoming the problem of bulk supply of halichondrin B. It was important to establish if *Lissodendoryx* sp. grown by aquaculture still produced the desired halichondrins. The investigation of sponge grown in aquaculture for halichondrin content formed another aspect of this project.

Chapter 2

epi-Isohomohalichondrin B

2.1 Introduction

Halichondrin-like species were first isolated from *Lissodendoryx* sp. in 1985 by Rob Lake.[57] Extraction of further material by Marc Litaudon enabled the structural assignment of one of these halichondrin species as isohomohalichondrin B (**1.28**).[86] It was found that this compound, if left in an NMR tube in deuterated methanol for several weeks, transformed itself into another compound 53-methoxyneoisohomohalichondrin B (**1.32**). This transformation was characterised by the shift of the H47 resonance from δ_H 3.27 to δ_H 3.25 (in CD_3OD) and the change in position of the resonances for methylenes 52, 54 and 55. At ambient temperature the transformation was complete in 6 weeks.[86] It was subsequently ascertained that this was transformation of isohomohalichondrin B (**1.28**) to the cyclic methoxy acetal **1.32**.

A joint venture between NIWA, New Zealand Pharmaceuticals and the Marine Chemistry Group was set up in 1994 to undertake the collection and extraction of one tonne of *Lissodendoryx* sp. with the aim of supplying a quantity of halichondrin B (**1.8**) to the National Cancer Institute (NCI) for preclinical trials. The extraction and purification of **1.8** was undertaken by NZ Pharmaceuticals with technical support in the form of P388 assays and ^{1}H NMR spectroscopy provided by the Marine Chemistry Group. A total of 310 mg of halichondrin B was supplied to the NCI for use in *in vivo* testing. All the side fractions from the purification of halichondrin B (**1.8**) were the property of the Marine Chemistry Group. Some of the homohalichondrin B (**1.25**) isolated from these side fractions was used in the experimental work described in this

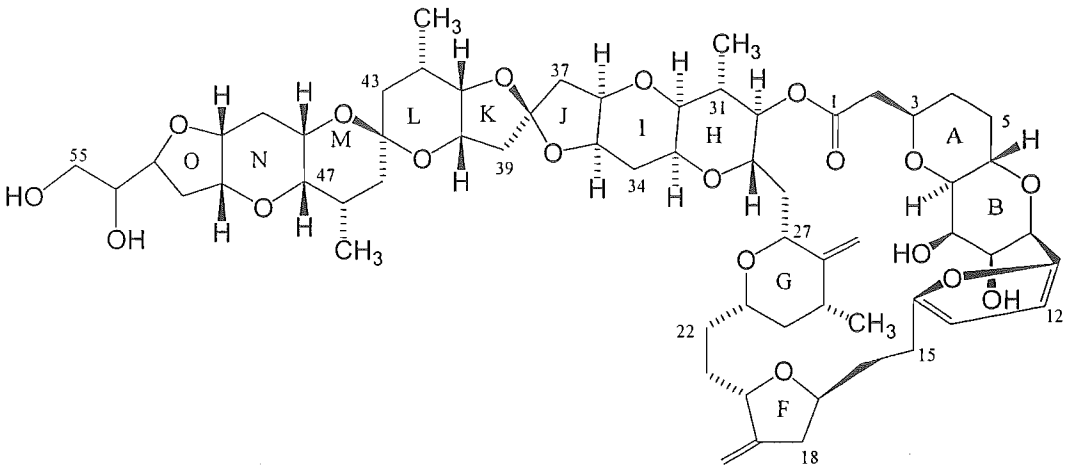
thesis. The isohomohalichondrin B (**1.28**) was supplied to PharmaMar, who hold the patent for **1.28** and were partners in the joint venture.

Fractions containing isohomohalichondrin B (**1.28**) were identified by comparing their HPLC chromatograms to those of authentic standards, and by characteristic ^{1}H NMR resonances. Gel permeation chromatography on LH20 eluted with CH_2Cl_2 was utilised to concentrated fractions of **1.28**. Analysis of the fractions from the LH20 chromatography by reverse phase (C18) HPLC indicated that, in addition to isohomohalichondrin B (**1.28**), a significant amount of a less polar halichondrin was present. A characteristic methoxy singlet at δ_H 3.23 in the ^{1}H NMR spectrum of some of these fractions confirmed the less polar component was **1.32**. An improved yield of **1.28** would be possible if the equilibrium position between these two halichondrins could be controlled in favour of **1.28**.

The previously observed interconversion of **1.28** to **1.32** in deuterated methanol, but not in deuterated chloroform containing 0.1% d_5-pyridine, gave an indication as to the equilibrium relationship between these two compounds. It had also been noted that the ^{1}H NMR spectrum of a sample of **1.32** kept in a $CDCl_3$ solution (without added C_5D_5N) slowly reverted to a spectrum characteristic of isohomohalichondrin B (**1.28**).[86] It was thought the addition of acid would facilitate the rapid attainment of equilibrium between **1.28** and **1.32**.

The treatment of halichondrins with acid had to be approached with some care, as a series of reactions by Joanne Hart had illustrated the vulnerability of halichondrins under acid conditions.[87] Treatment of homohalichondrin B (**1.25**) with 1.7 equivalents of TFA resulted in both furan formation, as a consequence of the opening of ether bonds at positions 9 and 14, and/or epimerisation at C38, shown for *epi*-homohalichondrin B furan (**2.1**).[87] The equilibrium relationship between some of these acid products was demonstrated. Homohalichondrin B (**1.25**) was shown to be stable to treatment with one equivalent of pyridinium *p*-toluene sulfonate (PPTS), as long as reaction times were

less than 72 hours. Treatment of **1.25** with a large excess of *p*-toluene sulfonic acid (PTSA) in CH_2Cl_2 for 48 hours had no affect.



epi-homohalichondrin B furan (**2.1**)

2.2 Formation and Isolation of **2.2**

Conversion of **1.32** to the more desirable isohomohalichondrin B (**1.28**) was attempted using dilute $HClO_4$. The conversion was initially followed by reverse phase (C18) analytical HPLC. A 100 μg fraction containing a mixture of **1.28** and **1.32** was treated with 5:1 $CH_3CN/HClO_4$ (120 μL, 0.035%). After 5 minutes, analysis of a 20 μL aliquot revealed the total absence of **1.32** in the sample. A short reaction time was preferential as further reaction resulted in the formation of another compound of similar polarity to **1.32**.

The reaction was repeated on a 1 mg scale to allow confirmation by ^1H NMR analysis. The reaction was halted after 5 minutes by diluting the sample with H_2O and applying it to a reverse phase (C18) cartridge. The acid was washed through with H_2O and the product stripped with CH_3CN and CH_2Cl_2. An increase in intensity of the characteristic triplet resonances of H54 and H55 of isohomohalichondrin B (**1.28**), a clean methyl region, and the absence of a methoxy singlet in the ^1H NMR spectrum of the product confirmed the transformation of **1.32** to **1.28**.

The reaction was scaled up for the conversion of larger amounts (200 mg) of **1.32** to **1.28**. The same concentration of acid (0.035%) was used as for the NMR trial. HPLC analysis of the product showed that in addition to the expected **1.28**, there was a second component **2.2** of similar polarity to **1.32**. The lack of a methoxy resonance in the ^{1}H NMR spectrum confirmed the component was not **1.32**. However, many resonances characteristic of the halichondrins were observed.

Conversion of a mixed sample of **1.32** and **1.28** to purely **1.28** was attempted, again using 0.035% $HClO_4$. The reaction time was reduced from 6 minutes to 1 minute, and the sample was loaded onto a C18 cartridge as quickly as was possible. The HPLC chromatogram of the product indicated that only **1.28** was present. This was supported by the ^{1}H NMR spectrum of the product, which was comparable to the ^{1}H NMR spectrum of a standard sample of **1.28**.

Reverse phase (C18) HPLC (90% CH_3CN/H_2O) was utilised to separate **2.2** (4.0 mg) from **1.28** (2.2 mg).

2.3 Structural Elucidation

The UV spectrum of **2.2** (eluting off the analytical C18 column at *ca* 1000 s in 90% CH_3CN/H_2O) indicated this compound contained no UV chromophore, with end absorption at λ 193 nm; a UV spectrum identical to isohomohalichondrin B (**1.28**). High resolution FABMS gave a parent ion corresponding to a molecular formula of $C_{60}H_{86}O_{19}$, isobaric with that of **1.28**.

The ^{1}H NMR spectrum of **2.2** is displayed in Figure 2.3.1. The absence of resonances at δ_H 5.95 and δ_H 6.19 together with the lack of a UV chromophore was indicative that a furan-type species, as observed in the homohalichondrin B series upon acid treatment,[87] had not been formed. A comparison of this spectrum with the ^{1}H NMR spectrum of **1.28** showed some differences. The doublet resonance of CH_3-42 had merged with that

of CH_3-31, the H32 resonance had moved upfield *ca* δ_H 0.1 and the isolated H41 triplet resonance was no longer evident.

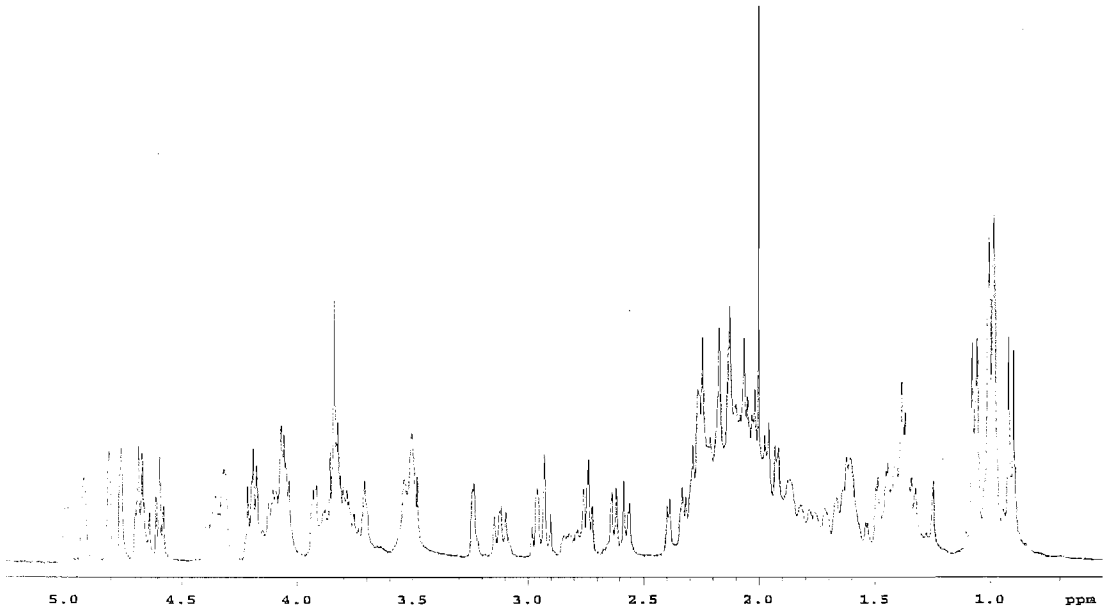


Figure 2.3.1 ^{1}H NMR Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

A full array of NMR experiments were performed on **2.2** *viz* COSY, 2D-TOCSY (mixing time 80 ms), HSMQC, HMBC, NOESY and APT (Figure 2.3.2) experiments. These data enabled the assignment of the majority of the ^{1}H and all of the ^{13}C NMR spectral data of **2.2** to be achieved. The ^{1}H and ^{13}C NMR data are collated in Table 2.3.1 and Table 2.3.2 respectively, and the important correlations from the 2D experiments are shown in Figure 2.3.3.

Table 2.3.1 ^{1}H NMR Data for *epi*-Isohomohalichondrin B (2.2)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.37	**H21**	1.40	**H39**	2.03
H2'	2.59	**H21'**	1.87	**H39'**	2.30
H3	3.88	**H22**	1.62	**H40**	3.93
H4	1.39	**H22'**	1.62	**H41**	3.84
H4'	1.76	**H23**	3.51	**H42**	2.25
H5	1.39	**H24**	1.05	**CH_3-42**	0.99
H5'	2.11	**H24'**	1.74	**H43**	1.37
H6	4.34	**H25**	2.20	**H43'**	1.48
H7	2.94	**CH_3-25**	1.07	**H45**	1.37
H8	4.31	**26=CH_2**	4.76	**H45'**	1.49
H9	4.06	**26'=CH_2**	4.81	**H46**	2.10
H10	4.19	**H27**	3.52	**CH_3-46**	0.91
H11	4.60	**H28**	1.94	**H47**	3.23
H12	4.68	**H28'**		**H48**	3.71
H13	1.95	**H29**	4.20	**H49**	1.84
H13'	2.13	**H30**	4.66	**H49'**	2.13
H15	1.62	**H31**	2.04	**H50**	3.51
H15'		**CH_3-31**	0.99	**H51**	3.81
H16	1.41	**H32**	3.12	**H52**	2.62
H16'	2.17	**H33**	3.77	**H52'**	2.93
H17	4.09	**H34**	2.06	**H54**	2.73
H18	2.26	**H34'**	2.25	**H54'**	2.73
H18'	2.80	**H35**	4.07	**H55**	3.83
19=CH_2	4.92	**H36**	4.14	**H55'**	3.83
19'=CH_2	4.99	**H37**	2.24		
H20	4.38	**H37'**	2.24		

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Figure 2.3.2 ^{13}C NMR Data for *epi*-Isohomohalichondrin B (**2.2**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	170.9	C21	29.4	C39	44.6
C2	40.4	C22	32.1	C40	71.8
C3	73.7	C23	74.9	C41	78.3
C4	30.7	C24	43.3	C-CH_3-42	25.4
C5	30.1	C-CH_3-25	36.0	C-CH_3-42	17.8
C6	68.2	C-CH_3-25	18.1	C43	36.9
C7	77.7	26C=CH_2	151.7	C44	97.0
C8	74.3	26C=CH_2	103.9	C45	37.4
C9	73.8	C27	73.4	C-CH_3-46	28.7
C10	76.5	C28	36.9	C-CH_3-46	17.0
C11	82.0	C29	71.2	C47	75.7
C12	81.0	C30	77.1	C48	66.3
C13	48.4	C-CH_3-31	36.3	C49	34.4
C14	110.0	C-CH_3-31	14.9	C50	66.3
C15	34.4	C32	77.3	C51	76.4
C16	28.2	C33	67.2	C52	45.2
C17	75.2	C34	29.7	C53	210.4
C18	38.7	C35	77.2	C54	46.0
19C=CH_2	151.3	C36	76.8	C55	57.9
19C=CH_2	104.3	C37	44.0		
C20	75.2	C38	113.9		

[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

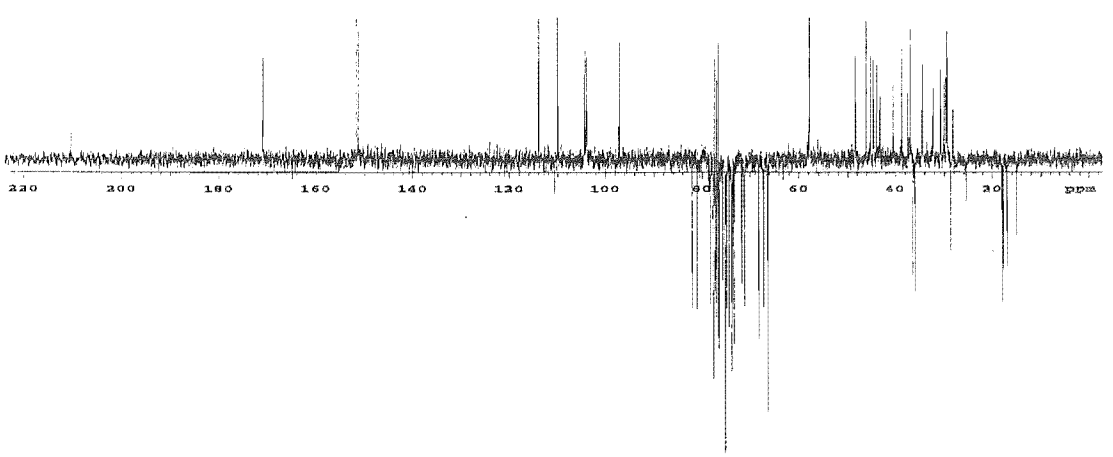
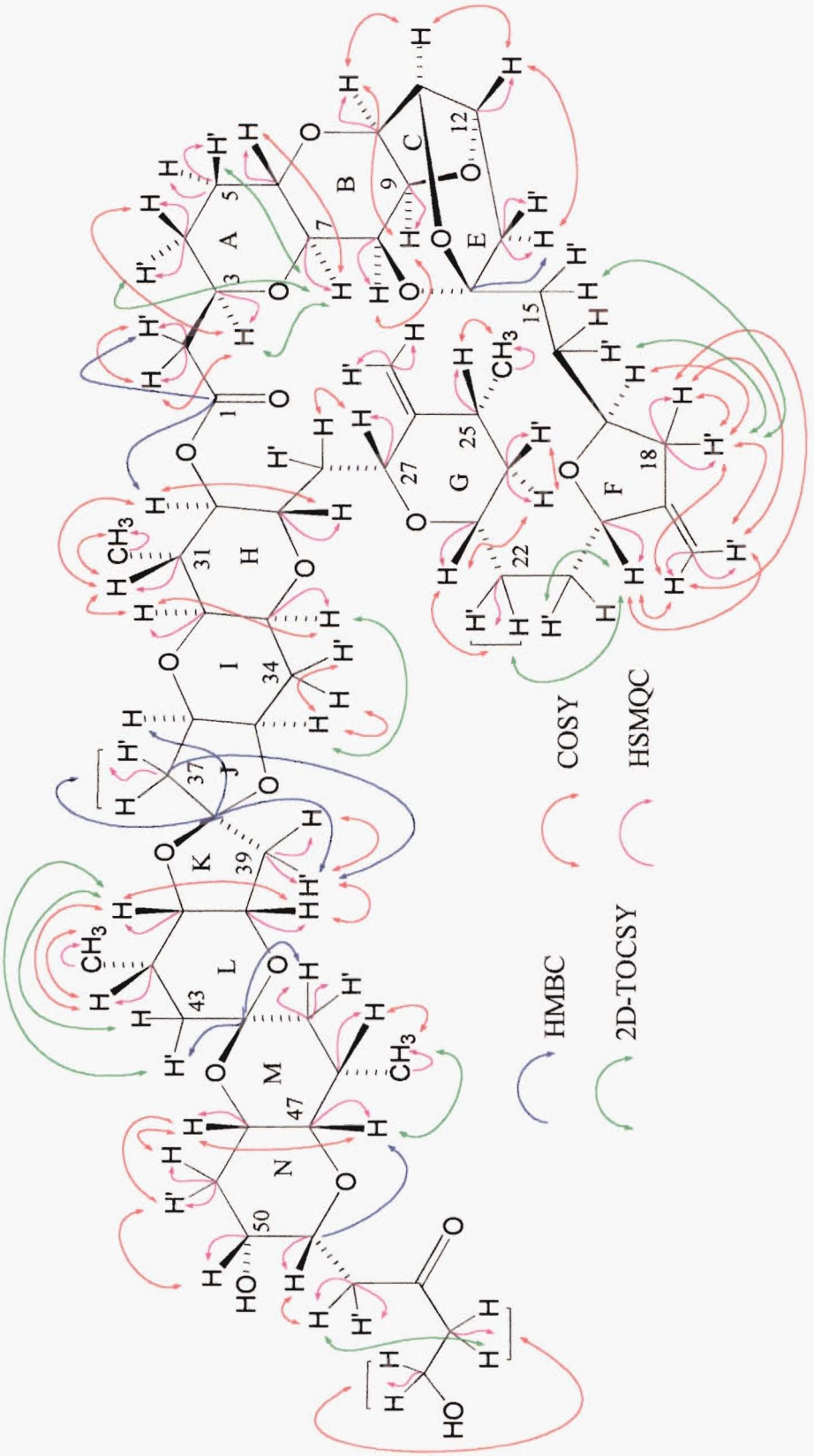


Figure 2.3.2 APT Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

Figure 2.3.3 *epi*-Isohomohalichondrin B - Important NMR Correlations



The COSY Experiment

The homonuclear COSY (COrrelation SpectroscopY) experiment was the first experiment used for the assignment of the protons in the ^{1}H NMR spectrum of *epi*-isohomohalichondrin B (**2.2**). From the COSY experiment, correlations between adjacent (geminal and vicinal) protons can be observed. The size of the coupling between protons is reflected in the intensity of correlation observed in the COSY spectrum. This is particular relevant to the assignment of ^{1}H NMR spectra of halichondrins. The halichondrin skeleton contains several *cis* fused rings. The coupling between protons at these ring junctions is small, and as a consequence COSY correlations between such protons are not always observed.

The use of the COSY experiment is illustrated in the assignment of the H2/2'-H13/13' protons (Figure 2.3.4). The isolated proton resonance of H11, and the partially obscured H7 and H2' protons provided a starting point for the assignment of the spin system. A correlation was observed between H11 and two methine protons H10 (δ_H 4.19) and H12 (δ_H 4.68). The H12 proton was coupled to one of the C13 methylene protons, H13 (δ_H 1.95). This proton, in turn, was coupled to the geminal H13' proton (δ_H 2.13). Moving back around the spin system, the H10 proton was coupled to H9 at δ_H 4.06. Coupled to the H9 proton was the H8 proton at δ_H 4.31. From the H7 proton a single correlation was seen to a methine proton. From this correlation, there were observed correlations to a methine proton (H9), and to methylene protons (H5, δ_H 1.39 and H5', δ_H 2.11). The observation of a single correlation from H7 was due to the similarity in chemical shift of the H8 (δ_H 4.31) and H6 protons (δ_H 4.34). The overlapping nature of the methylene region of the COSY spectrum made it impossible to observe the correlations between the methylene protons of C4 and C5. Working back toward H5 from H2', an intense correlation was observed between the H2' proton (δ_H 2.59) and the geminal H2 proton (δ_H 2.37). A correlation was also observed from the H2' proton to a methine proton (H3, δ_H 3.88). In turn, the H3 proton was correlated to another methylene proton (H4, δ_H

1.39). The remaining protons in the C1-C14 spin system were assigned from the TOCSY spectrum.

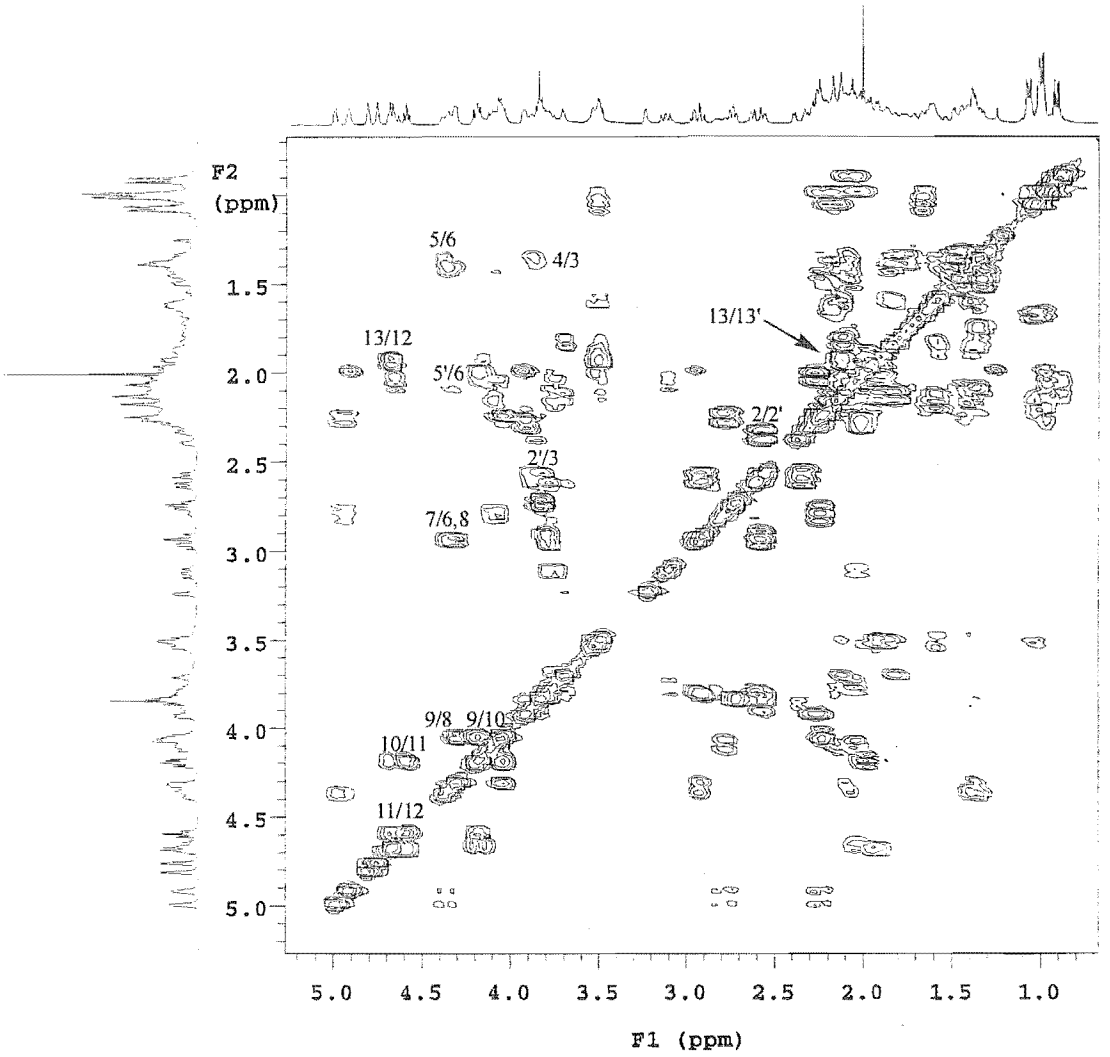


Figure 2.3.4 COSY Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

The TOCSY Experiment

The TOCSY (TOtal Correlation SpectroscopY) experiment is an extension of the COSY experiment that allows the connectivity of protons within a spin system to be probed. The appearance of a TOCSY correlation is dependent on the magnitude of the coupling constant for the transfer of magnetisation through the spin system. The length of the mixing time, during which the magnetisation is transferred, also has an effect on the TOCSY signal. A longer mixing time will allow transfer of magnetisation further

through a spin system, but the advantage of this has to be weighed against a decrease in the signal to noise ratio as relaxation occurs.

The 2D-TOCSY spectrum for *epi*-isohomohalichondrin B (**2.2**) is displayed in Figure 2.3.5. The assignment of the C1-C14 spin system is used once more to illustrate the use of the TOCSY experiment in the assignment of the ^{1}H NMR spectrum of **2.2**.

Looking along the H11 cross section (Figure 2.3.6), correlations can be seen to H12, H8, H10, H9, H13' and H13. From both H7 and H2' TOCSY correlations are seen to H5' (δ_H 2.11), H4' (δ_H 1.76) and a methylene proton at δ_H 1.39 (H4 and H5).

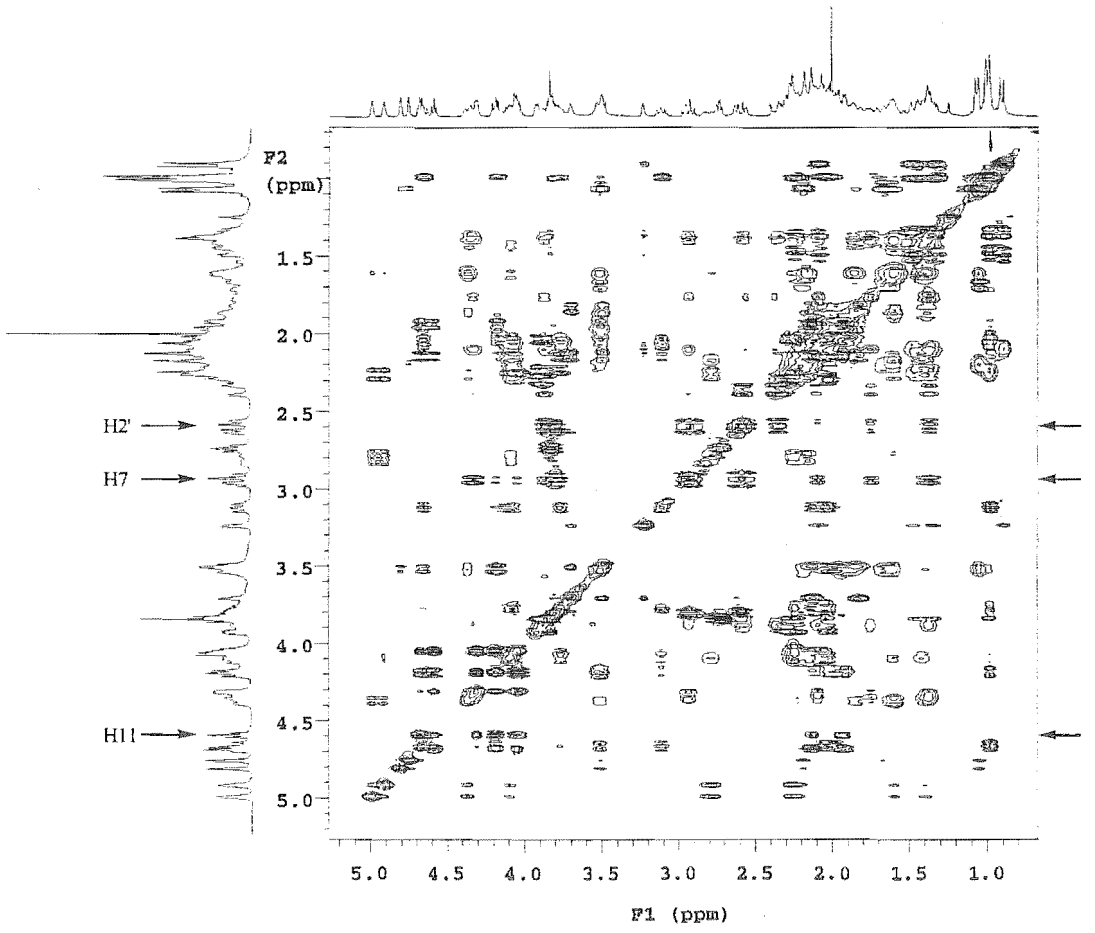


Figure 2.3.5 2D-TOCSY Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

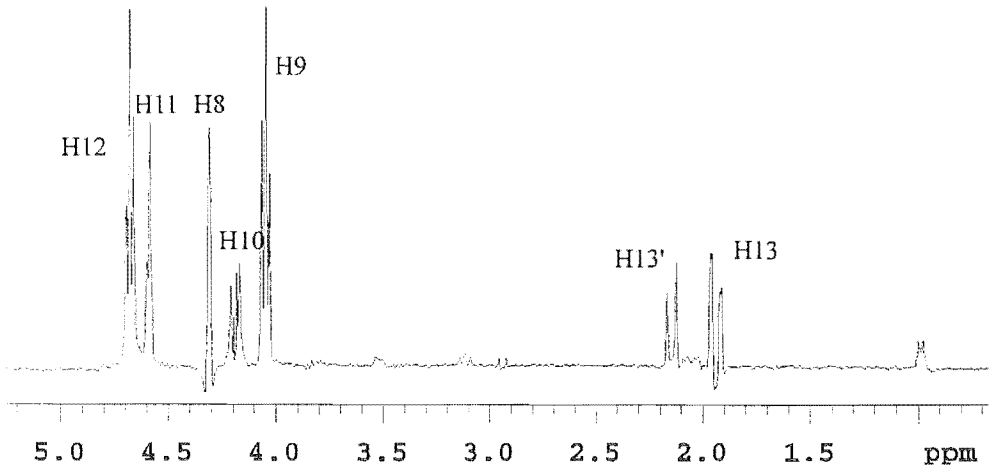


Figure 2.3.6 Cross-section of 2D-TOCSY Spectrum through H11

The 1D-TOCSY is a related experiment that is performed by selectively irradiating individual signals in the ^{1}H NMR spectrum. Generally this experiment requires the signals to be isolated, but it may be possible to dereplicate a 1D-TOCSY obtained by the irradiation of two overlapping signals. The 1D-TOCSY spectrum is essentially the same as a cross-section through the 2D-TOCSY spectrum. The major advantage of the 1D-TOCSY experiment is the reduction in time required to obtain a given signal to noise ratio. The resolution, and hence coupling information, of the 1D-TOCSY experiment is superior to that of the 2D-TOCSY experiment. An array of mixing times may be used for a given irradiated proton to give a range of 1D-TOCSY spectra that enable a picture of the connectivity relative to the irradiated proton to be established. An example of the 1D-TOCSY experiment with variable mixing times is given in Section 6.3.2.7.

The HSQC Experiment

The HSQC (Heteronuclear Single Quantum Coherence) experiment is a proton-detected experiment that indicates carbons directly attached to protons. This experiment was particularly useful in the indirect assignment of the ^{13}C spectrum of halichondrin derivatives where there was inadequate mass for a ^{13}C or APT spectrum to be obtained. The HSMQC (Heteronuclear Single and Multiple Quantum Coherence) experiment was

used to obtain the same one bond carbon to proton information as the HSQC experiment for some of the earlier work described in this thesis.

Correlations to methylene protons in the COSY and TOCSY spectra are sometimes difficult to see due to the complex, overlapping nature of this region of the spectrum, so the HSQC experiment gives a useful indication of the chemical shift equivalence or otherwise of methylene protons. In the assignment of *epi*-isohomohalichondrin B (**2.2**), the HSMQC experiment showed that the C39 carbon was correlated to methylene protons of distinct chemical shift (δ_H 2.03 and δ_H 2.30), whereas the protons correlated to the C37 carbon had equivalent chemical shifts (δ_H 2.24) (Figure 2.3.7).

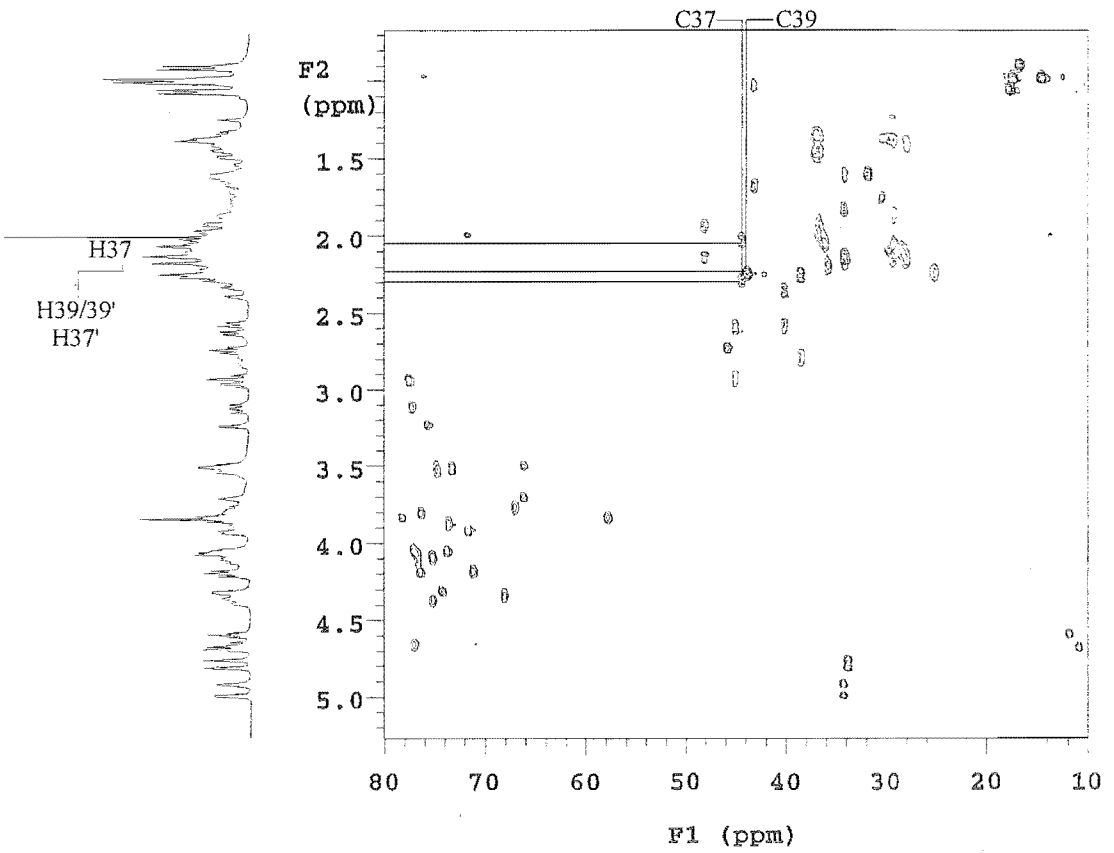


Figure 2.3.7 HSMQC Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

The HMBC Experiment

The HMBC (Heteronuclear Multiple Bond Coherence) experiment shows correlations to carbons two and three bonds removed from a proton. This experiment is useful in

establishing connectivity. In cases where there was insufficient material for obtaining a ^{13}C spectrum, this experiment offered the only means of assigning the ^{13}C chemical shift of the non-protonated carbons of the halichondrin skeleton, *viz* the lactone C1, and the spiro ketal C14, C38 and C44 carbons. HMBC correlations traverse heteroatoms, which is particularly useful in the case of the halichondrins for the observation of three bond correlations through ether bonds. The HMBC spectrum of *epi*-isohomohalichondrin B (**2.2**) is displayed in Figure 2.3.8.

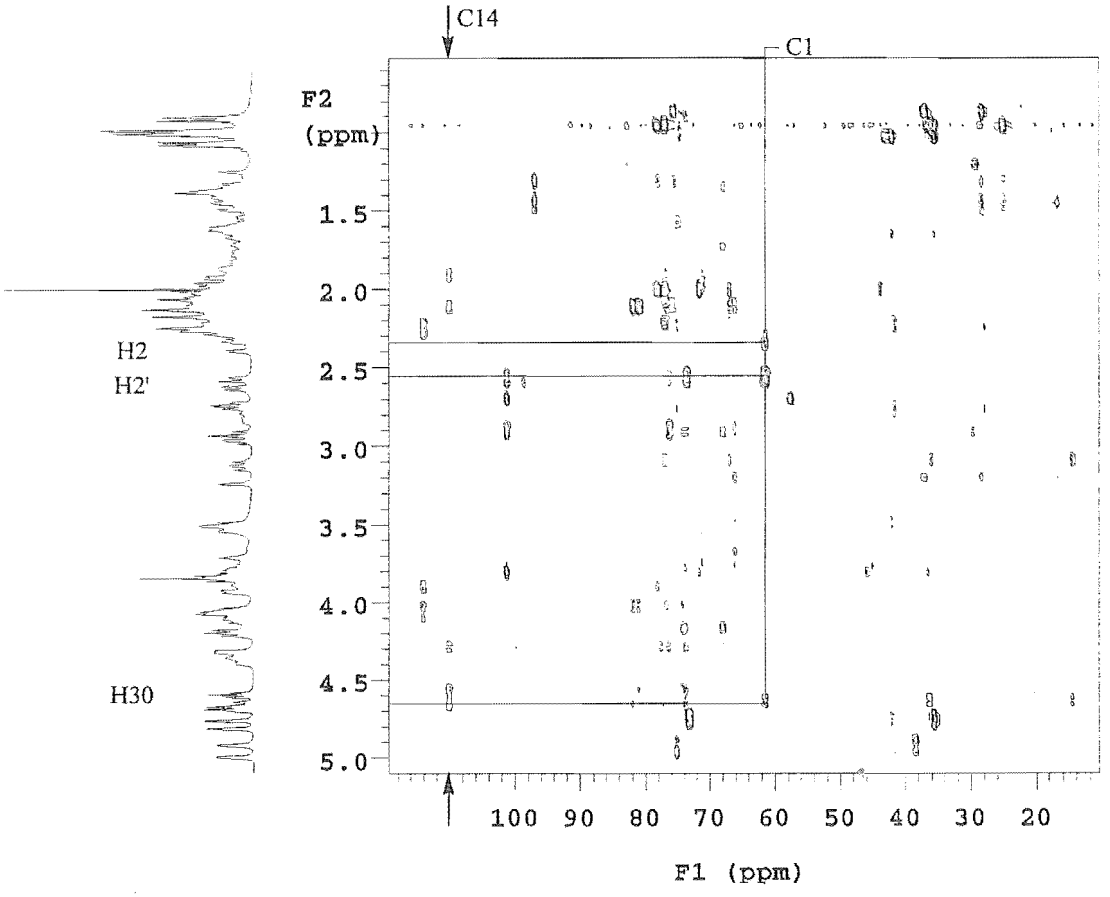


Figure 2.3.8 HMBC Spectrum of *epi*-Isohomohalichondrin B (**2.2**)

A cross section through the quaternary C14 carbon shows correlations to H12, H11 (through oxygen), H8, H13' and H13 (Figure 2.3.9). The HSQC and HMBC experiments are run using a narrowed carbon window to reduce the time required to obtain the equivalent signal to noise ratio. A reasonably wide window was used for acquiring the HMBC spectrum of **2.2**, but the typical spectral window used was δ_C 80 to

δ_C 10. Correlations to the lactone carbon (C1, δ_C 170.9) from H30, H2 and H2' appear as a foldback to δ_C 60.9.

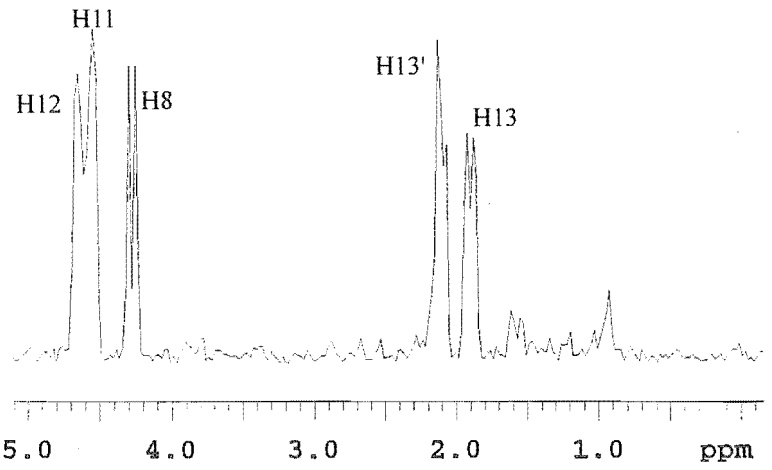


Figure 2.3.9 Cross-section of HMBC Spectrum through C14

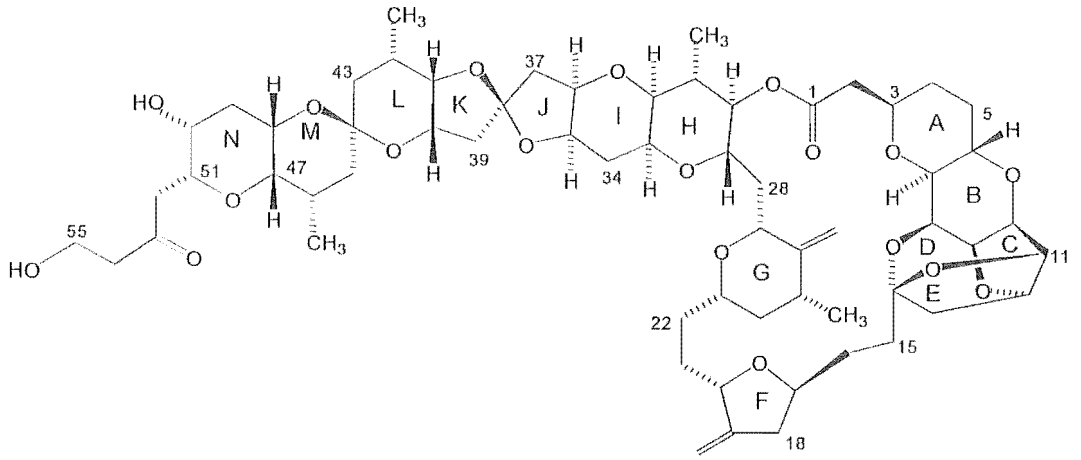
Using the experiments described above to assign the ^{1}H and ^{13}C NMR spectra of **2.2** it was possible to confirm the connectivities and NMR shifts around the C1-C30 and C47-C55 regions were identical to those of **1.28**. A COSY correlation from the isolated H32 resonance to δ_H 3.78 allowed the assignment of the chemical shift of the H33 proton. A correlation to δ_H 2.06 in the COSY spectrum from the H33 resonance allowed the assignment of the chemical shift of the H34 proton. The chemical shift of the H34' proton (δ_H 2.25) was assigned from a correlation in the COSY spectrum from the geminal H34 resonance. From the H32 and the H33 proton resonances a correlation was seen in the 2D TOCSY spectrum to δ_H 4.07. This resonance was assigned to the H35 proton. An HMBC correlation from the C38 spiro carbon resonance (δ_C 113.9) to δ_H 4.14 allowed the assignment of the chemical shift of H36. Further HMBC correlations were observed from C38 to H35, H40 and to unassigned proton resonances at δ_H 2.24 and δ_H 2.30. In the HSQC spectrum, the proton at δ_H 2.30 and another resonance at δ_H 2.03 were correlated to a carbon at δ_C 44.6. A COSY correlation from the H40 resonance (δ_H 3.93) to δ_H 2.30 allowed the assignment of H39' and hence of H39 (δ_H 2.03). The remaining resonance (δ_H 2.24) was attributed to H37. A COSY correlation from the H40 resonance to a proton resonating at δ_H 3.84 allowed the

assignment of the chemical shift of the H41 methine proton. A correlation from the CH_3-42 resonance to a proton resonating at δ_H 2.25 allowed the assignment of the chemical shift of the H42 proton. The 2D-TOCSY showed correlations from the H41 methine resonance to protons resonating at δ_H 1.37 and δ_H 1.48. These resonances were assigned to the H43 and H43' protons. An HMBC correlation from the C44 spiro carbon to this pair of methylene proton resonances supported this assignment. A COSY correlation from the CH_3-46 (0.91) resonance to δ_H 2.10 allowed the assignment of the chemical shift of H46. H45 and H45' were assigned from the observation of a correlation from δ_H 0.91 (CH_3-46) to δ_H 1.37 and δ_H 1.49.

The assigned NMR data for **2.2** were compared to those of **1.28**.[59] Significant chemical shift differences ($\geq \delta_H$ 0.5) were observed for most protons in the region of C32-C46. Carbon chemical shift differences were centred on C38.

In terms of specific chemical shift differences, the H41 proton (δ_H 3.84) exhibited a large downfield shift of δ_H 0.20 relative to **1.28**. The H39 (δ_H 2.03) and H39' (δ_H 2.30) protons appeared as two separate resonances. This was in contrast to the H39/H39' methylene pair of **1.28** that had a chemical shift of δ_H 2.22. The converse was true of the H37/H37' methylene pair. The H37 and H37' ^{1}H NMR resonances of **2.2** were equivalent (δ_H 2.24) whereas they appeared as separate signals (δ_H 1.92 and δ_H 2.37) in the ^{1}H NMR spectrum of isohomohalichondrin B (**1.28**). The C38 spiro carbon exhibited a change in ^{13}C NMR chemical shift of δ_C 1.5 downfield.

The observed changes in ^{1}H and ^{13}C NMR chemical shifts described above are consistent with epimerisation at C38 to give *epi*-isohomohalichondrin B (**2.2**).



epi-isohomohalichondrin B (**2.2**)

2.4 Acid Equilibria

The equilibrium character of the C38 epimerisation was investigated. It was envisaged the addition of a trace amount of acid would catalyse the rapid achievement of equilibrium between **2.2** and **1.28**. The determination of the position of equilibrium between **1.28** and **2.2** was desirable, as it would allow the possibility of obtaining further **1.28** to be assessed.

Treatment of **2.2** with $HClO_4$ (0.035%) was monitored by reverse phase (C18) HPLC, eluted with 90% CH_3CN/H_2O. The relative amount of **1.28** in the sample increased to a maximum of 50% after 3 hours. Analysis of the reaction after 4 hours confirmed the equilibrium ratio of **1.28**:**2.2** was 1:1. The acid conditions were apparently not strong enough to cause opening of the trioxadecalin ring system as observed in the earlier work on homohalichondrin B.[87]

2.5 Biological Activity

The biological activity of **2.2** was evaluated in the *in vitro* P388 assay. Control samples of **1.28** and **1.32** were assayed in the same batch to allow a valid comparison of activity

data. These data are cited in Table 2.1 along with those for the equivalent acid products in the homohalichondrin B series.

Table 2.1 Cytotoxicity of Related Halichondrins

Compound	**P388 IC_{50} (ng/mL)**
isohomohalichondrin B (**1.28**)	**0.20**
53-methoxyneoisohomohalichondrin B (**1.32**)	2.9
epi-isohomohalichondrin B (**2.2**)	3.4
homohalichondrin B (**1.25**)	0.22
epi-homohalichondrin B (**2.3**)	15.0

From the above data it can be concluded that epimerisation at C38 has a detrimental affect on the cytotoxicity of the halichondrin series. The C38 epimer **2.2** is 17-fold less active relative to the parent **1.28**. This reduction in cytotoxicity is similar to the equivalent result for the homohalichondrin B epimer **2.3** and the parent homohalichondrin B (**1.25**).

2.6 Discussion

Conditions for the conversion of **1.32** to **1.28** have been established. It was found that the most important consideration was the time of acid contact with the halichondrin mixture. The largest scale reaction was performed using the same overall concentration, but less molar equivalents of perchloric acid than the NMR trial reaction and a subsequent successful reaction. The notable difference was reaction time and the time taken to load the diluted reaction mixture onto a larger C18 column. Subsequent large scale (*ca* 100 mg) transformations utilised a 1 g reverse phase (C18) cartridge for acid removal.[88] This allowed the diluted reaction solution to be loaded rapidly onto the C18 column and the acid washed out with water. The epimer **2.2** was fully characterised, and the equilibrium relationship between **2.2** and **1.28** established.

Chapter 3

Aquacultured *Lissodendoryx* sp.

3.1 Introduction

The options for halichondrin B supply are collection of native sponge, synthesis or aquaculture. The native supply of *Lissodendoryx* sp. is inadequate to cater to the needs of clinical supply in a sustainable manner. As yet synthesis is not a commercially viable option for the production of halichondrin B for clinical use, although efforts in this direction are ongoing.[73] The aquaculture of *Lissodendoryx* sp. appears to be the most viable method of producing the quantities of halichondrin B required should it enter into clinical use as a chemotherapeutic agent.

Sponge aquaculture has been recorded as early as the 18th century in the Mediterranean.[89] However sponge aquaculture for the production of biologically active metabolites is still in its infancy. Sponge cultivation essentially consists of cutting pieces from a sponge and attaching these explants to some type of submerged anchor to allow regeneration to occur. Factors affecting sponge growth include depth, light intensity, temperature, water movement and nutrient conditions.[90]

The successful aquaculture of *Lissodendoryx* sp. by NIWA is discussed in Section 1.2.4.3. The investigation and quantification of halichondrin content in *Lissodendoryx* sp. grown in aquaculture is presented in this chapter.

3.2 Sponge Production

Sponge explants were grown in the Marlborough Sounds (Beatrix Bay) and Banks Peninsula (Akaroa). At each location two sites, between fifty to one hundred metres apart, were used. Sponge samples were grown in tiered lanterns at shallow (-5 to -7 m), mid depth (-10 to -15 m) and deep (-25 to -30 m) positions. Sixty nine sponge samples classified in terms of site number, depth and tier were harvested from the aquaculture sites at Beatrix Bay and Akaroa. To allow halichondrin detection to be feasible, the samples were combined on the basis of depth at each of the two sites, giving three Beatrix Bay samples (shallow, mid depth and deep) and two Akaroa samples (shallow and deep).

3.3 Extraction and Purification

Each of the five sponge samples was blended with MeOH, stirred overnight, filtered, and then repeatedly extracted with MeOH. The MeOH/H_2O ratio was adjusted to 4:1 before partitioning against petroleum ether (PE). With the exception of the Beatrix Bay deep extract, the aqueous MeOH partitions were evaporated *in vacuo*, and the residue coated onto Celite and subjected to reverse phase (C18) "flash" chromatography.[91] A step gradient elution from 1:1 MeOH/H_2O to MeOH then 1:1 MeOH/CH_2Cl_2 was utilised, before stripping the column with CH_2Cl_2 and MeOH. In the case of the Beatrix Bay deep sample, the MeOH was evaporated from the aqueous methanol partition to afford an aqueous extract. This extract was partitioned against EtOAc in order to reduce the mass of the sample, before evaporating the solvent *in vacuo*, coating the residue onto Celite and purifying by reverse phase (C18) chromatography as described above. All three Beatrix Bay samples exhibited identical separation profiles, with activity concentrated in a single fraction (90% MeOH/H_2O). In contrast, the two extracts originating from Akaroa had reduced activity spread over several fractions.

The P388 active fractions in each series were combined and subjected to further fractionation by size exclusion chromatography on LH20 eluted with CH_2Cl_2. TLC (DIOL, 1% MeOH/CH_2Cl_2) of the active fractions from LH20 revealed the presence of the desired halichondrin B (**1.8**), homohalichondrin B (**1.25**) and isohomohalichondrin B (**1.28**). However, it was not possible to observe halichondrin peaks in the HPLC chromatograms of the active fractions due to coeluting UV-active components.

In an attempt to remove these impurities one of the active fractions was subjected to a trial purification using normal phase (DIOL) chromatography. The DIOL cartridge (250 mg) was eluted with benzene, 1:1 benzene/CH_2Cl_2, 1:99 MeOH/CH_2Cl_2, 1:9 MeOH/CH_2Cl_2 and MeOH. TLC (DIOL, 1% MeOH/CH_2Cl_2) indicated that the nonpolar impurities eluted with benzene and the halichondrins were eluted with 1:1 benzene/CH_2Cl_2 (largely **1.28**) and CH_2Cl_2 (**1.8** and **1.25**). As a consequence of this result the remaining active fractions were combined within each series and subjected to purification by normal phase (DIOL) chromatography. The elution scheme was subsequently simplified to benzene, CH_2Cl_2, 1:9 MeOH/CH_2Cl_2 and 1:1 MeOH/CH_2Cl_2. The halichondrins were concentrated into the CH_2Cl_2 fraction in each case.

3.4 Analysis

The five samples were analysed by reverse phase (C18) HPLC for halichondrin content. This involved comparing the retention time of halichondrin-type (end absorption only) peaks to those of standard samples for halichondrin B (**1.8**), homohalichondrin B (**1.25**) and isohomohalichondrin B (**1.28**). A typical HPLC chromatogram of the purified aquaculture extracts is displayed in Figure 3.4.1.

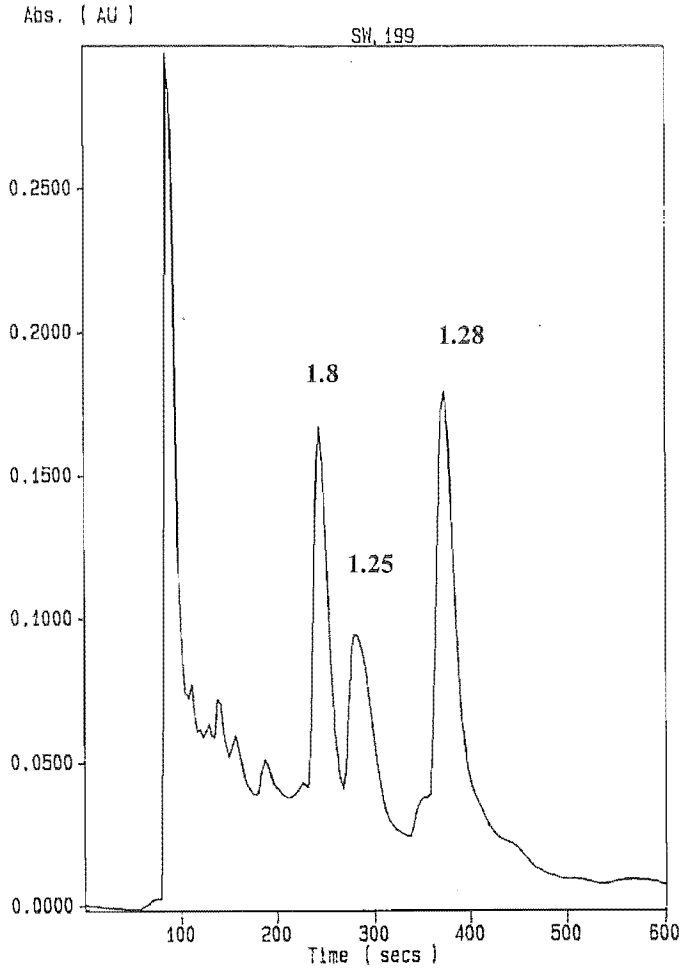


Figure 3.4.1 HPLC Chromatogram of a Purified *Lissodendoryx* Extract

The mass of the individual halichondrins in each sample was determined by comparing the integral of halichondrin peaks with that of a known amount of halichondrin B. The estimation of isohomohalichondrin B (**1.28**) content in both the Akaroa and Beatrix Bay deep samples was hindered by the coelution of a UV-active impurity. The presence of **1.28** in these samples was shown by TLC (DIOL, 1% MeOH/H_2O). The mass of **1.28** in the Beatrix Bay sample was estimated indirectly from the halichondrin ratio exhibited by the two other samples originating from this site. It was not possible to estimate the amount of **1.28** contained in the Akaroa sample.

The results are shown in Figure 3.4.2. In addition to the aquacultured sponge, a sample of native (Kaikoura) *Lissodendoryx* sp. was extracted and purified in the same manner as described for the aquacultured sponge samples in Section 3.5. This control extract

enabled a comparison of halichondrin content to be made between the native and the aquacultured sponge.

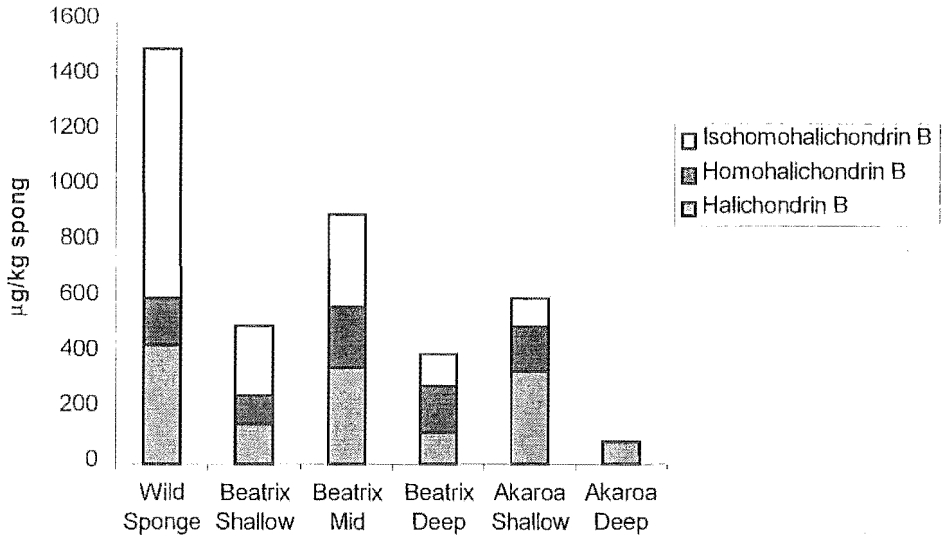


Figure 3.4.2 Halichondrin Levels in *Lissodendoryx* Samples

Two conclusions can be drawn immediately. Halichondrins, and more especially halichondrin B (**1.8**) are produced by *Lissodendoryx* sp. grown in aquaculture. The levels of total halichondrins produced by aquacultured sponge are less than those of the native sponge. It is interesting to note that in the cases of the Akaroa shallow sponge and the Beatrix Bay mid depth sponge, the levels of halichondrin B (**1.8**) are not dissimilar to those of the native sponge. This result is encouraging, and may indicate it is possible to influence the production of individual halichondrins by manipulation of the aquaculture environment.

Halichondrin production is not the only variable to be considered in determining optimum conditions for the aquaculture of *Lissodendoryx* sp. In order for the production of halichondrins by harvest of aquacultured sponge to be viable, sponge

survival and growth rates must also be maximised. A comparison between halichondrin production (μg per kg wet weight of sponge) and sponge yield is illustrated in Figure 3.4.3.

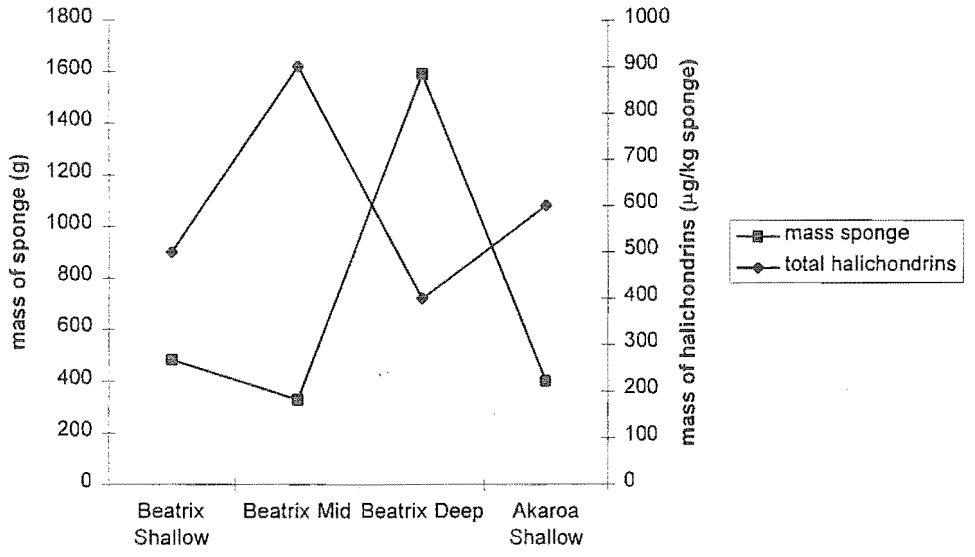


Figure 3.4.3 Comparison of Halichondrin Production against Sponge Yield

The highest rate of halichondrin production is negatively correlated with growth. A possible explanation for this observation is that halichondrin production increased in sponges suffering from stress.

3.5 Discussion

This is the first proof that extracts of *Lissodendoryx* sponge grown in aquaculture produce halichondrins. The quantity of halichondrin, and the ratio of the three halichondrins detected varied depending on the site and depth at which the sponge was grown. NIWA are examining the reasons for these differences and developing techniques for increasing both sponge growth rate and halichondrin content. Land-based aquaculture trials are also being undertaken to examine the factors responsible for fast growth and halichondrin production.[92] It is hoped that these experiments will

facilitate better understanding of the processes involved in the biosynthesis of halichondrin B.

Chapter 4

Terminal Haptens

4.1 Haptens - An Introduction

In the event that halichondrin B proceeds to further trials, an effective means of detecting halichondrins in biological matrices will be required. The extreme potency of halichondrin B requires any selected method to have the ability to detect halichondrins down to the pg level. The development of an Enzyme Linked Immunosorbent Assay (ELISA) was perceived as the best means of achieving these requirements.

In the case of low molecular weight compounds such as the halichondrins, the development of an immunoassay normally requires synthesis of a protein-reactive derivative (hapten) of the compound to allow conjugation to an immunogenic carrier protein. To allow for the possibility of both class-specific halichondrin assays (distinction between the A, B and C families) and congener specificity (distinction between halichondrin B and other congeners in the B family) the site of conjugation has to be carefully selected. It is generally observed that the portion of a hapten most distal to the conjugation site most profoundly affects antibody recognition.[93] Immunogenic proteins conjugated at the terminal site of any member of the B series of halichondrins should elicit antibodies that recognise the macrocyclic portion of the molecule (*ie* will distinguish between the A, B and C families). Similarly, antibodies elicited by immunogenic proteins conjugated distal to the terminal site of a B series halichondrin

should discriminate between the B series congeners (halichondrin B, homohalichondrin B, isohomohalichondrin B, etc.).

The haptens were prepared in collaboration with the Hawaii Biotechnology Group Inc (HBG). At the time the haptens were synthesised it was expected that HBG would prepare the hapten carrier conjugates and undertake the development of a halichondrin immunoassay. The linkers used in the preparation of haptens were recommended by HBG.[94] The use of the hydroxy reactive *p*-maleimidostyryl isocyanate (PMSI) linker (PMSI, **4.1**)[a] to synthesise haptens from homohalichondrin B (**1.25**) and isohomohalichondrin B (**1.28**) is described in Section 4.2. The closely related *p*-maleimidophenyl isocyanate (PMPI, **4.2**) linker was used to create haptens from halichondrin B (**1.8**) and homohalichondrin B (**1.25**) and is discussed in Section 4.3. The terminal diol moiety of **1.25** was cleaved to furnish norhomohalichondrin B (**4.13**). This enabled the synthesis of an alternatively linked hapten, while still incorporating the common maleimide functionality, which is discussed in Section 4.5.

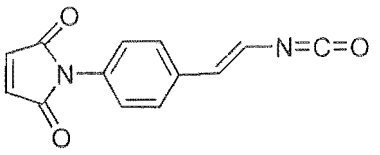
4.2 PMSI Linker-derived Haptens

4.2.1 Introduction

PMSI (**4.1**) consists of a hydroxy reactive isocyanate functionality, a styryl spacer group and a sulfhydryl-reactive maleimide moiety. The maleimide hapten will react readily with the sulfhydryl residues of proteins thiolated with 2-iminothiolane to yield a stable thioether linkage.

---

[a] The donation of PMSI by Hawaii Biotechnology Group Inc. is gratefully acknowledged.



PMSI (**4.1**)

4.2.2 Reaction of Isohomohalichondrin B (1.28) with PMSI (4.1)

Isohomohalichondrin B (**1.28**, 2.5 mg) was initially treated with **4.1** for 8 hours. TLC (DIOL, 3% MeOH/CH_2Cl_2) of the reaction mixture showed that both **1.28** and **4.1** were still present. After reaction for a further 19 hours resonances characteristic of **1.28** and **4.1** could still be seen in the ^{1}H NMR spectrum of the reaction mixture. Isohomohalichondrin B (**1.28**) and **4.1** were kept in the NMR tube in 0.1% $C_5D_5N/CDCl_3$ (*ca* 150 μL) for 4 days. Changes to key resonances in the ^{1}H NMR spectrum of the reaction mixture after 4 days indicated the reaction had gone to completion. The characteristic H54/54' and H55 triplet resonances of **1.28** were no longer observed. The H2' resonance was observed as a clean doublet of doublets, with the previously overlapping H52' resonance moving downfield by δ_H 0.21. The most obvious evidence for reaction of **4.1** having occurred was the shift in the resonance for the styryl protons from δ_H 6.61 and δ_H 6.43 to δ_H 5.92 and δ_H 7.18 respectively.

Chromatography of the product mixture on LH20, using CH_2Cl_2 as the eluent, was used to isolate hapten **4.3** (2.0 mg) from PMSI-derived side products.

The ^{1}H NMR spectrum of **4.3** is displayed in Figure 4.2.1. A minimum of NMR experiments were performed on **4.3** to enable the location of key resonance changes *viz* HSMQC and 2D-TOCSY (mixing time 100 ms) experiments. These data were used to assign the majority of the ^{1}H NMR and ^{13}C NMR resonances of **4.3**. The ^{1}H and ^{13}C NMR data are collated in Table 4.2.1 and Table 4.2.2 respectively, and the important correlations from the 2D experiments are shown in Figure 4.2.2.

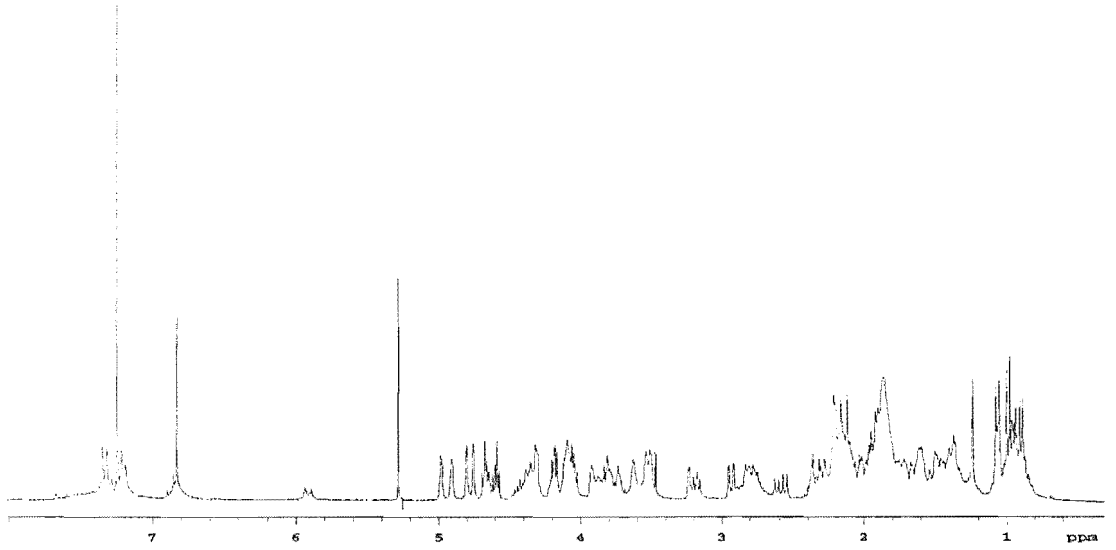
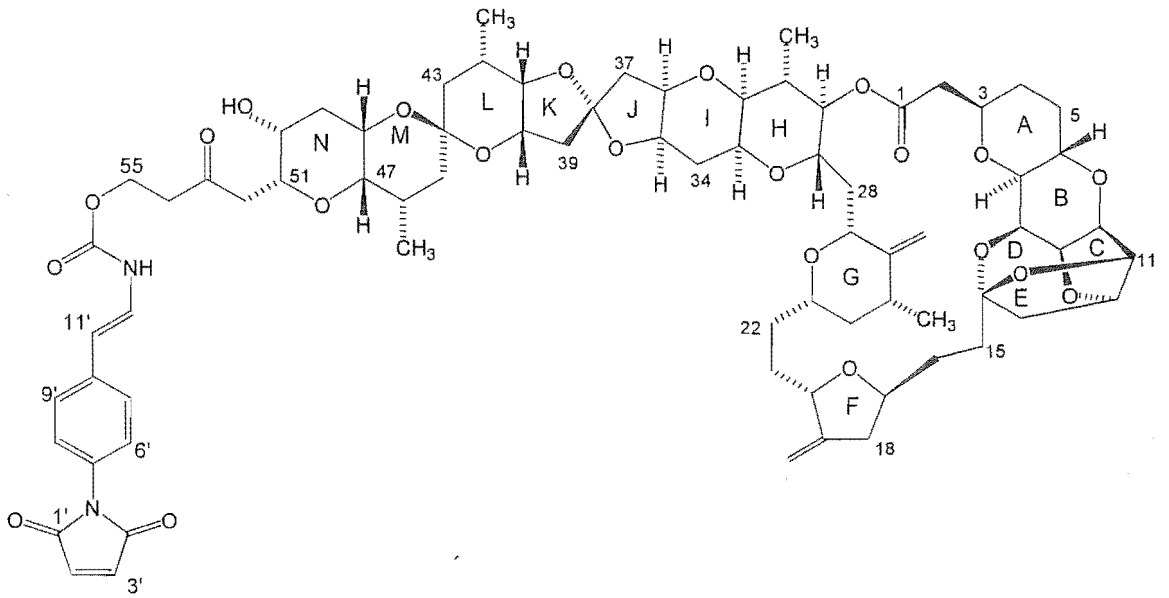


Figure 4.2.1 ^{1}H NMR spectrum of Isohomohalichondrin B PMSI Hapten (**4.3**)



isohomohalichondrin B PMSI hapten (**4.3**)

Table 4.2.1 ^{1}H NMR Data for Isohomohalichondrin B PMSI Hapten (**4.3**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H22**	1.61	**H42**	2.29
H2'	2.59	**H22'**	1.61	**CH_3-42**	0.95
H3	3.88	**H23**	3.51	**H43**	1.34
H4	1.37	**H24**		**H43'**	1.53
H4'	1.74	**H24'**	1.69	**H45**	1.49
H5	1.41	**H25**	2.20	**H45'**	1.49
H5'	2.10	**CH_3-25**	1.06	**H46**	2.16
H6	4.35	**26=CH_2**	4.76	**CH_3-46**	0.89
H7	2.94	**26'=CH_2**	4.80	**H47**	3.23
H8	4.31	**H27**	3.52	**H48**	3.74
H9	4.05	**H28**		**H49**	1.84
H10	4.19	**H28'**		**H49'**	2.13
H11	4.59	**H29**	4.19	**H50**	3.52
H12	4.68	**H30**	4.65	**H51**	3.82
H13	1.94	**H31**	2.02	**H52**	2.81
H13'	2.15	**CH_3-31**	0.99	**H52'**	2.81
H15	1.61	**H32**	3.18	**H54**	2.83
H15'		**H33**	3.80	**H54'**	2.83
H16	2.16	**H34**	1.78	**H55**	4.38
H16'	1.42	**H34'**	2.14	**H55'**	4.45
H17	4.09	**H35**	4.10	**H2'**	6.83
H18	2.26	**H36**	4.09	**H3'**	6.83
H18'	2.79	**H37**	1.90	**H6'**	7.23
19=CH_2	4.91	**H37'**	2.34	**H7'**	7.34
19'=CH_2	4.99	**H39**	2.21	**H9'**	7.34
H20	4.37	**H39'**	2.21	**H10'**	7.23
H21	1.40	**H40**	3.93	**H11'**	5.92
H21'		**H41**	3.63	**H12'**	7.18

[a] The symbol ' represents the less shielded proton of a geminal pair.

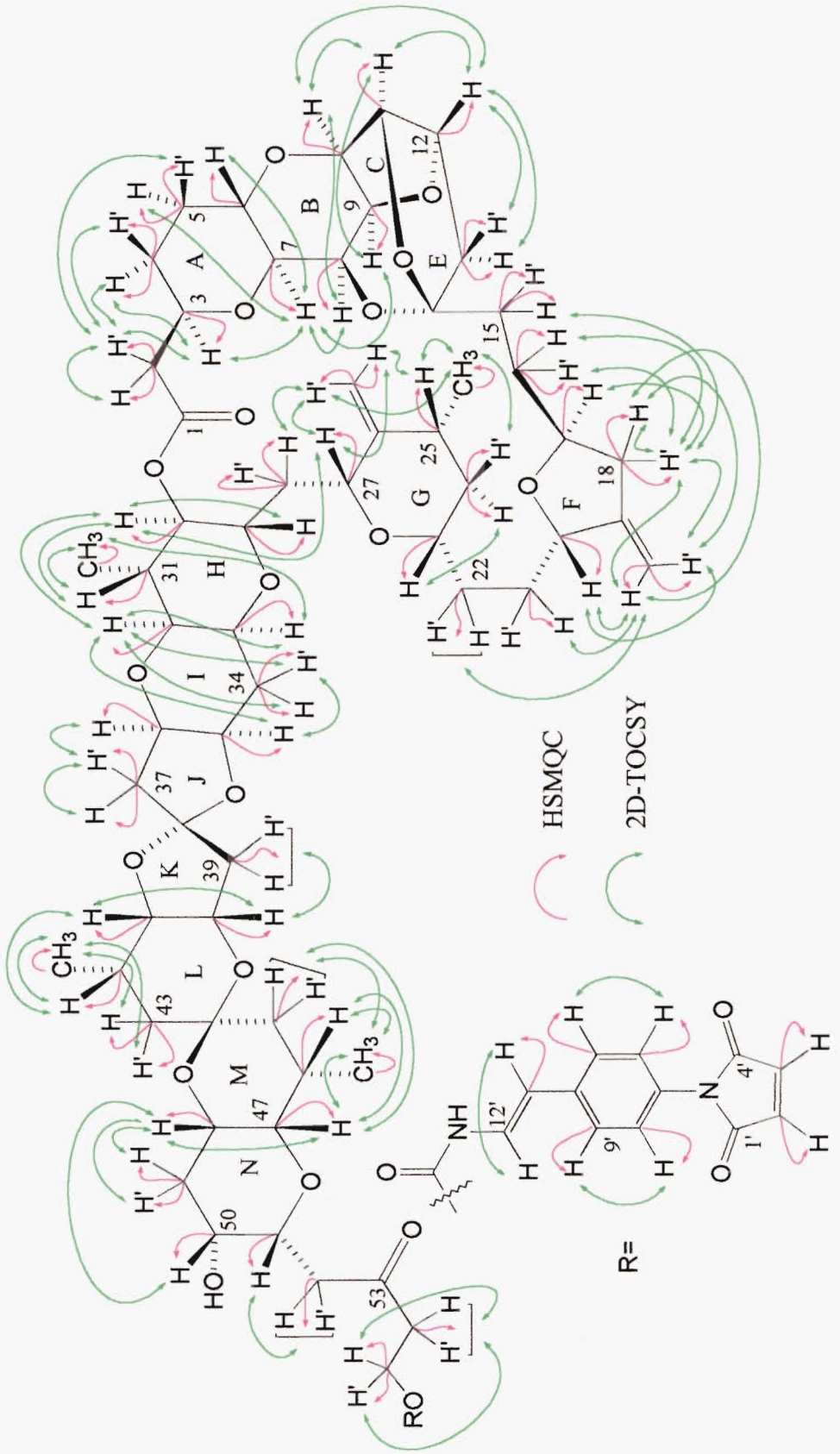
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 4.2.2 ^{13}C NMR Data for Isohomohalichondrin B PMSI Hapten (**4.3**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1		**C-CH_3-25**	35.9	**C-CH_3-46**	28.5
C2	40.3	**C-CH_3-25**	17.8	**C-CH_3-46**	16.8
C3	73.6	**26C=CH_2**		**C47**	75.9
C4	30.5	**26C=CH_2**	104.1	**C48**	66.4
C5	29.9	**C27**	73.5	**C49**	34.3
C6	68.2	**C28**	36.8	**C50**	66.1
C7	77.6	**C29**	71.0	**C51**	76.2
C8	74.3	**C30**	76.8	**C52**	45.4
C9	73.8	**C-CH_3-31**	36.6	**C53**	
C10	76.5	**C-CH_3-31**	14.8	**C54**	42.5
C11	82.0	**C32**	77.4	**C55**	60.7
C12	81.1	**C33**	66.4	**C1'**	
C13	48.2	**C34**	29.0	**C2'**	134.2
C14		**C35**	75.2	**C3'**	134.2
C15	34.3	**C36**	76.2	**C4'**	
C16	28.1	**C37**	43.4	**C5'**	
C17	75.2	**C38**		**C6'**	126.1
C18	38.6	**C39**	42.4	**C7'**	125.8
19C=CH_2		**C40**	71.1	**C8'**	
19C=CH_2	104.4	**C41**	79.0	**C9'**	125.8
C20	75.3	**C-CH_3-42**	25.7	**C10'**	126.1
C21	29.3	**C-CH_3-42**	17.5	**C11'**	109.6
C22	32.0	**C43**	36.9	**C12'**	
C23	74.7	**C44**		**C13'**	
C24	43.2	**C45**	37.1		

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from an HSMQC spectrum at 300 MHz.

Figure 4.2.2 Isohomohalichondrin B PMSI Hapten – Important NMR Correlations

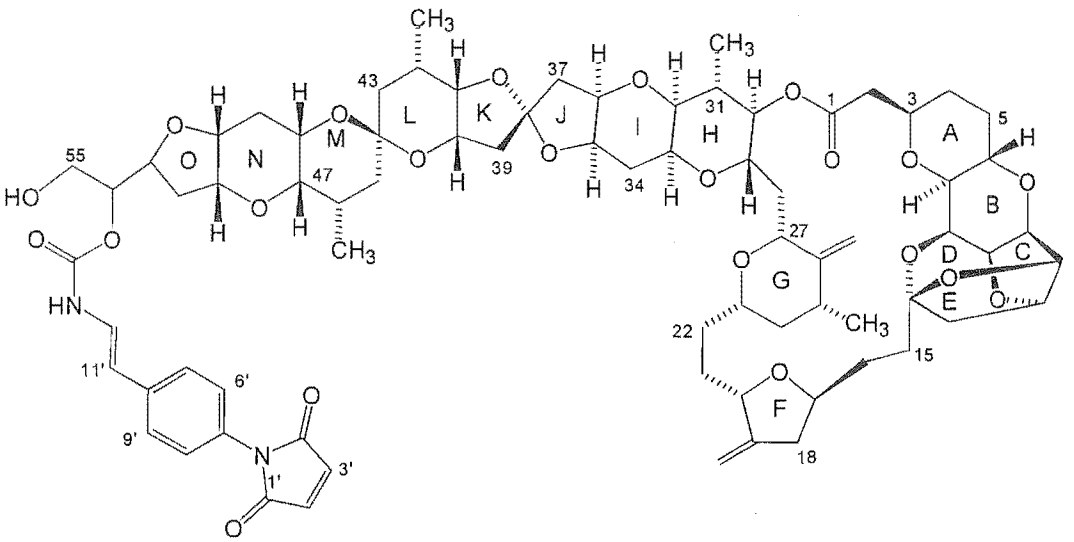


The ^{1}H and ^{13}C NMR spectral data in the region of the A-N rings of hapten **4.3** were consistent with those of isohomohalichondrin B (**1.28**). The most notable differences in chemical shift for **4.3** relative to **1.28** were observed for the H52/52' and H55/55' methylene resonances. The H55/55' proton resonances, observed at a coincident chemical shift in **1.28** (δ_H 3.86) were seen as 2 distinct resonances for **4.3** (δ_H 4.38, δ_H 4.45). The H52/52' protons appeared as a single resonance (δ_H 2.81). The assignment of the H52/52' proton resonances was confirmed by the observation of a TOCSY correlation between the H51 resonance (δ_H 3.82) and the resonance at δ_H 2.81. The equivalent protons in **1.28** had separate resonances (δ_H 2.93 and δ_H 2.62 respectively). The H54/54' methylene proton resonances (δ_H 2.83) were also different to those of the parent **1.28** (δ_H 2.74). A significant change in ^{13}C chemical shift data was apparent for C54, with a shift difference relative to C54 of **1.28** of δ_C 3.6 upfield. The C55 resonance of **4.3** at δ_C 60.7 was downfield compared to the C55 resonance of **1.28** (δ_C 57.8). The chemical shift of C52 for **4.3** was not significantly different to that of the parent **1.28**, confirming that the C52 hydroxy group did not react with **4.1**. The downfield styryl proton resonance (δ_H 7.18) was located from a 2D-TOCSY correlation to this resonance from the isolated upfield styryl proton resonance at δ_H 5.92.

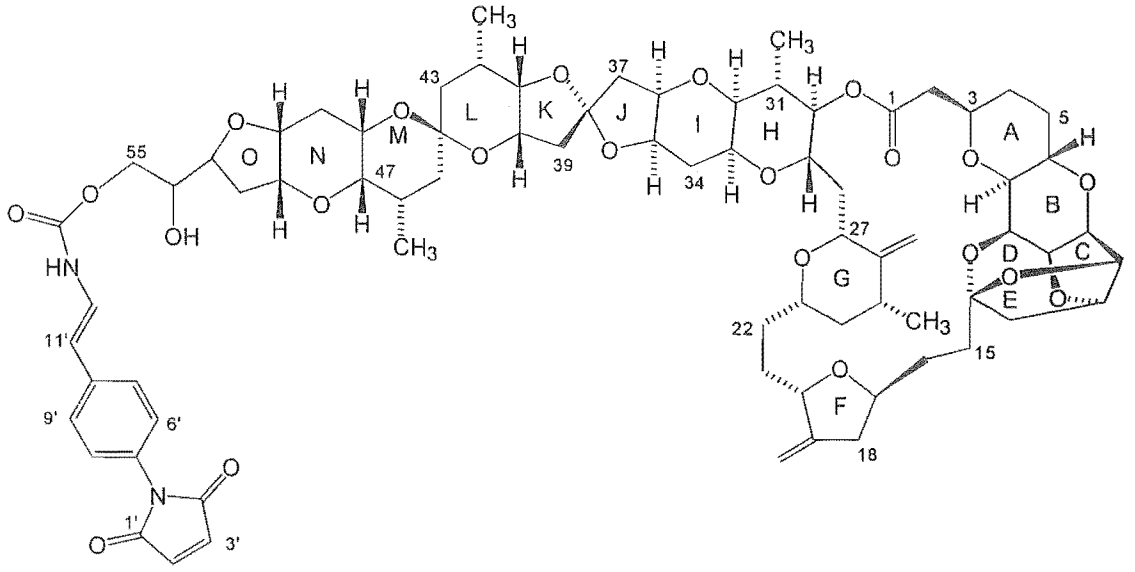
4.2.3 Homohalichondrin B and PMSI

Homohalichondrin B (**1.25**, 1.3 mg) was treated with an equimolar quantity of **4.1**. The reaction was monitored by ^{1}H NMR spectroscopy. It was deemed to be complete after 5 days, at which time the styryl proton resonances of **4.1**, and the H55/55' resonance of **1.25** were no longer observed in the ^{1}H NMR spectrum of the reaction mixture. The presence of several maleimide proton resonances was indicative of the formation of a mixture of products. Reverse phase (C18) HPLC using 60% CH_3CN/H_2O as the mobile phase was used to separate the product mixture into four fractions. The most polar fraction was identified as the starting material **1.25** by comparison of its retention time and ^{1}H NMR spectrum to those of an authentic sample. Of the remaining three

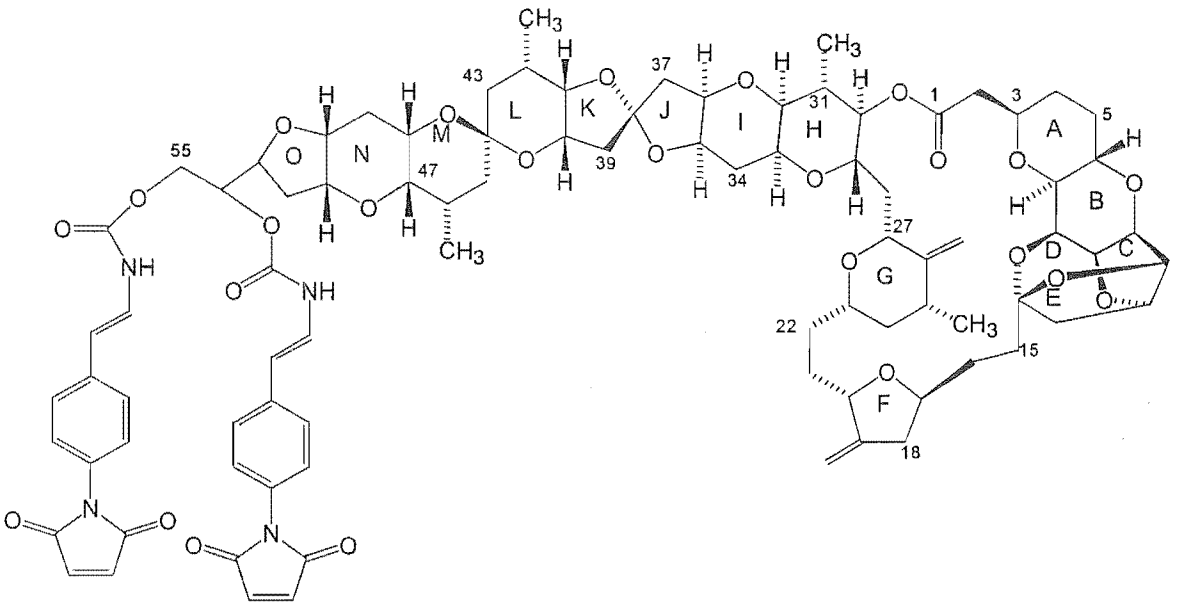
fractions, two were similar in polarity (**4.4**, **4.5**), and one was notably less polar (**4.6**). The molecular formula of the less polar **4.6** was determined as $C_{87}H_{102}O_{25}N_4$ by HRFABMS. This was consistent with the addition of **4.1** linkers to both hydroxy groups of **1.25**. The polarity of **4.6** relative to **1.25** was also consistent with the masking of the polar diol of **1.25**. The isobaric **4.4** and **4.5** were found to have a molecular formula of $C_{74}H_{94}O_{22}N_2$ by HRFABMS. This molecular formula was consistent with the attachment of a single **4.1** linker at the C54 hydroxy group, or the C55 hydroxy group of **1.25**. The lack of material precluded the use of 2D NMR experiments to assign the H54 and H55/55' resonances. The major differences in the ^{1}H NMR spectrum of **4.5** compared to that of **1.25** were in the region of δ_H 4.00-4.25. The distinct H55 resonance (δ_H 3.69) was not observed. The predominant product **4.5** is likely to be the result of reaction of the primary C55 hydroxy group of **1.25** with **4.1** (see also Section 4.3.2).



homohalichondrin B PMSI hapten (**4.4**)



homohalichondrin B PMSI hapten (**4.5**)



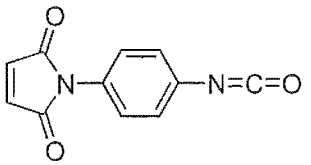
homohalichondrin B PMSI hapten (**4.6**)

The ratio of haptens formed was determined by the integration of their peaks in the HPLC chromatogram of the product mixture. The ratio of **4.4**:**4.5**:**4.6** was determined to be 11:84:5. This is comparable to the ratio of products obtained in the reaction of **1.25** with the phenyl linker **4.2** (Section 4.3.2).

4.3 PMPI Linker-derived Haptens

4.3.1 Introduction

Within the structure of PMPI (**4.2**) is an hydroxy-reactive isocyanate functionality, a phenyl spacer group and a sulfhydryl reactive maleimide moiety. The maleimide haptens will react readily with sulfhydryl residues in proteins thiolated with 2-iminothiolane to form a stable thioether linkage.

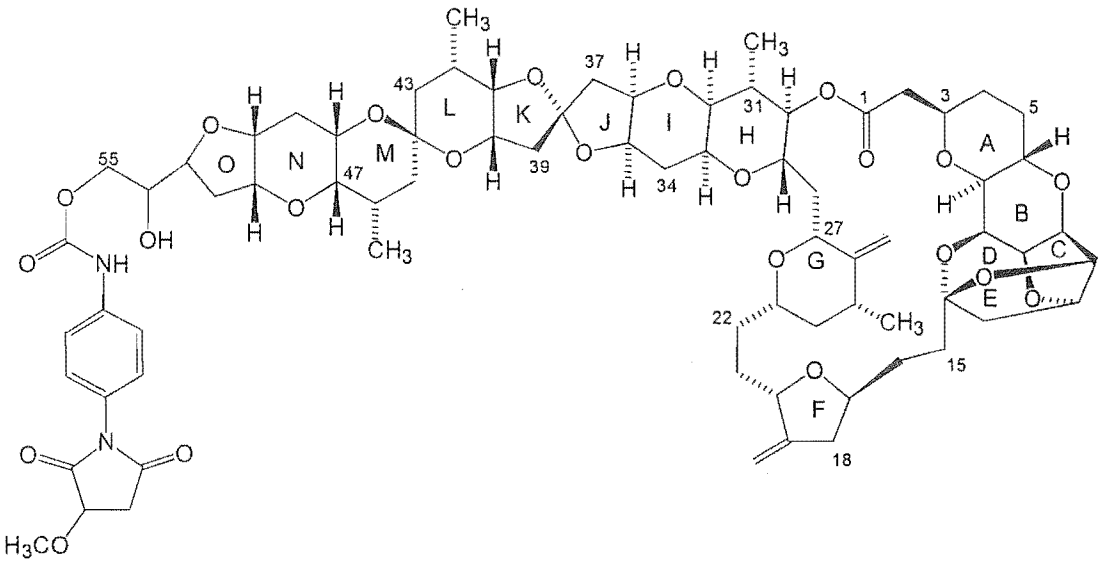


PMPI (**4.2**)

4.3.2 Reaction of Homohalichondrin B and PMPI

In order to obtain predominantly monoconjugated product, an equimolar amount of PMPI (**4.2**) was reacted with homohalichondrin B (**1.25**, 0.9 mg). The progress of the reaction was monitored by ^{1}H NMR spectroscopy. No further change in the ^{1}H NMR spectrum of the reaction mixture was seen after 64 hours. That more than one product had been formed was evident from the complex nature of the methyl region of the spectrum. Semipreparative HPLC using a reverse phase (C18) column eluted with 60% CH_3CN/H_2O was utilised to separate the product mixture into four fractions. The most polar fraction was identified as starting material **1.25** by comparison of its retention time and ^{1}H NMR spectrum to those of an authentic sample of **1.25**. The major product **4.7** eluted at 600 s, just after one of the minor products. The third, minor product was considerably less polar, with a retention time of *ca* 1200 s. The relative polarities of the products indicated it was likely, as expected, that the more polar fractions were monoconjugated **1.25**, and that the much less polar fraction was diconjugated **1.25**.

The appearance of a doublet of doublets (δ_H 6.33), and a decrease in intensity of the characteristic maleimide proton resonance a *ca* δ_H 6.8 was observed in the ^{1}H NMR spectrum of the major product **4.7**. This indicated that the maleimide olefin was no longer intact. Analysis by reverse phase (C18) HPLC showed **4.7** was more polar than the product peak collected under identical conditions. The molecular formula of **4.7** ($C_{73}H_{96}N_2O_{23}$) determined by HRFABMS was consistent with the addition of MeOH to the double bond of the maleimide. This was supported by the observation of a methoxy resonance (δ_H 3.49) in the ^{1}H spectrum of **4.7**. The addition of MeOH seems most likely to have occurred when MeOH was used for transferring purified fractions into storage vials. In hindsight this was obviously a very poor choice of solvent.



methanol addition product (**4.7**)

The addition of an equimolar amount of PMPI (**4.2**) to **1.25** (2.5 mg) was repeated. The reaction was deemed to be complete after 5 minutes, when no further changes were observed in the ^{1}H NMR spectrum of the reaction mixture. The HPLC chromatograph of the mixture was comparable to that obtained for the previous reaction. Semipreparative reverse phase (C18) HPLC was used to separate the four components.

4.3.2.1 Characterisation of **4.8**

The ^{1}H NMR spectrum (Figure 4.3.1) of the least polar component **4.8** contained two maleimide proton resonances, consistent with the addition of **4.2** to both hydroxy groups of **1.25**. There were two notable additions to the ^{1}H NMR spectrum relative to that of the parent compound; a multiplet resonating at δ_H 5.14 and a resonance at *ca* δ_H 4.4. Partial ^{1}H NMR assignments were made from spectral data collected from a 2D-TOCSY (mixing time 80 ms) experiment. The assigned data are listed in Table 4.3.1 and the important 2D-TOCSY correlations are shown in Figure 4.3.2.

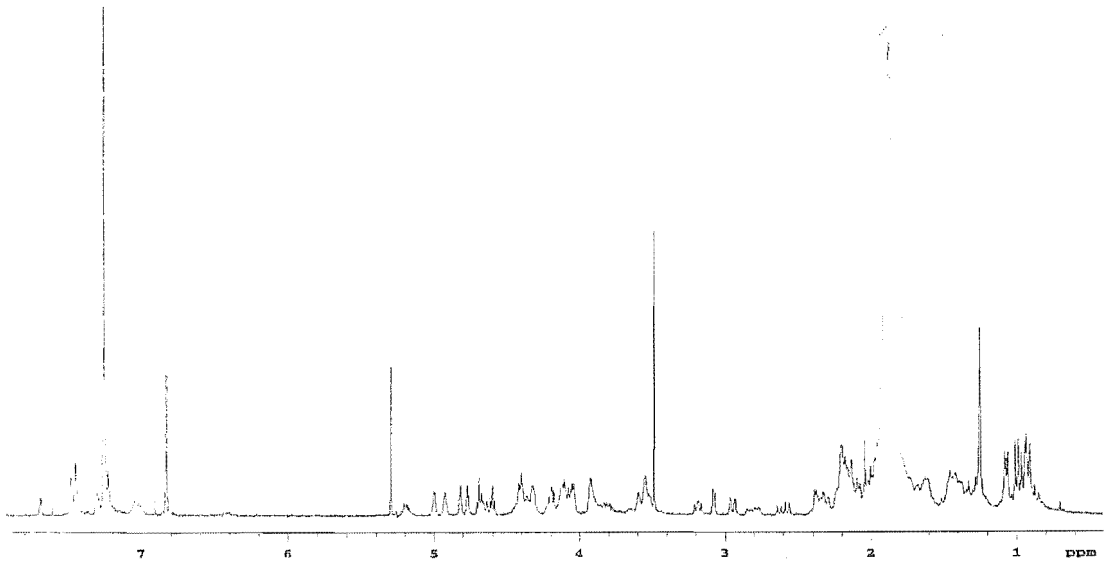


Figure 4.3.1 ^{1}H NMR Spectrum of Homohalichondrin B PMPI Hapten (**4.8**)

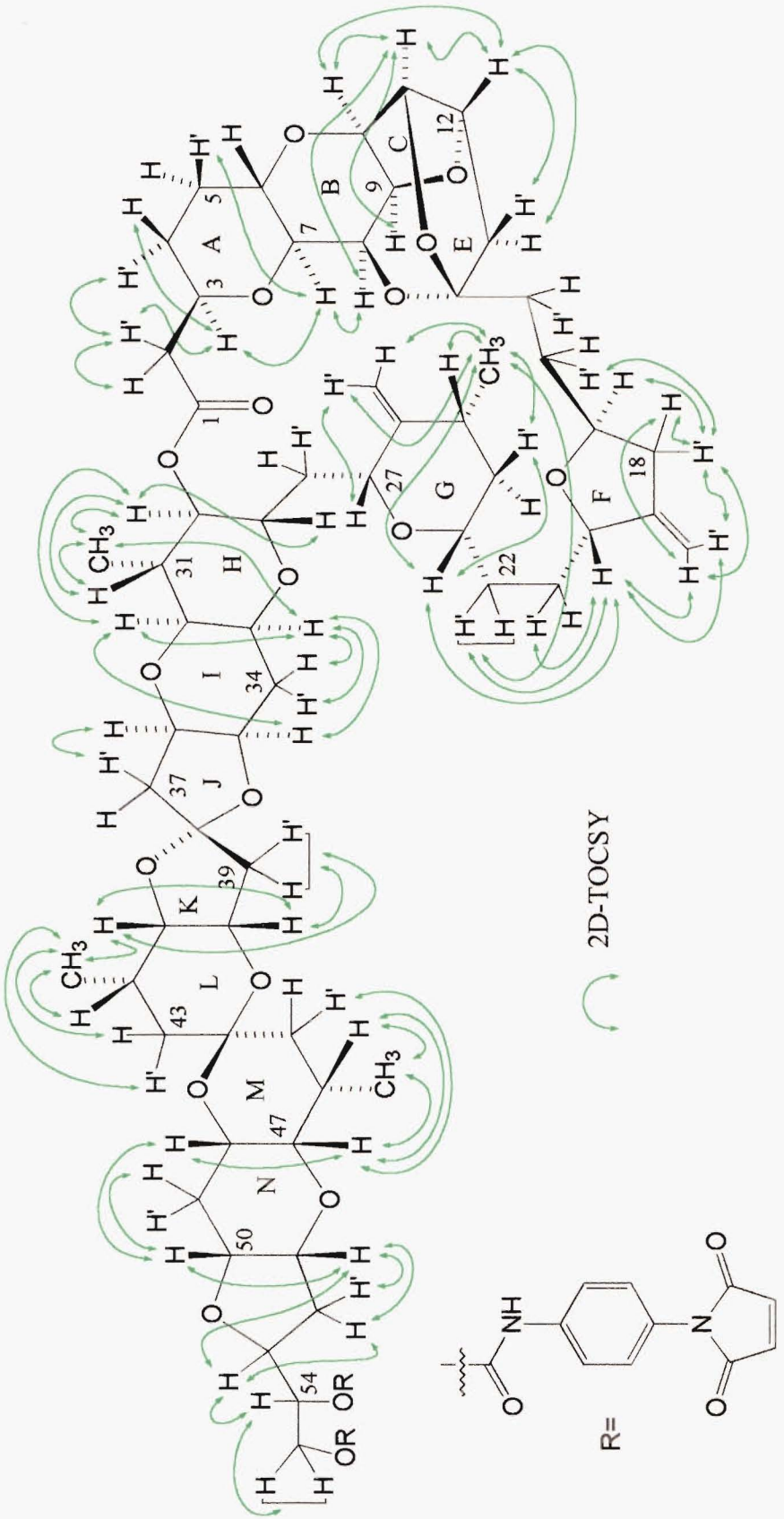
Table 4.3.1 ^{1}H NMR Data for Homohalichondrin B PMPI Hapten (**4.8**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.35	**H21**		**H39**	2.20
H2'	2.60	**H21'**	1.88	**H39'**	2.20
H3	3.88	**H22**	1.61	**H40**	3.92
H4		**H22'**	1.61	**H41**	3.59
H4'	1.73	**H23**	3.53	**H42**	2.35
H5		**H24**		**CH_3-42**	0.93
H5'	2.10	**H24'**	1.69	**H43**	1.30
H6		**H25**	2.21	**H43'**	1.46
H7	2.95	**CH_3-25**	1.07	**H45**	
H8	4.32	**26=CH_2**	4.77	**H45'**	1.44
H9	4.05	**26'=CH_2**	4.82	**H46**	2.17
H10	4.19	**H27**	3.53	**CH_3-46**	0.92
H11	4.60	**H28**		**H47**	3.08
H12	4.69	**H28'**		**H48**	3.54
H13	1.94	**H29**	4.19	**H49**	1.79
H13'	2.15	**H30**	4.66	**H49'**	
H15		**H31**	2.03	**H50**	3.91
H15'		**CH_3-31**	1.00	**H51**	4.04
H16		**H32**	3.19	**H52**	1.87
H16'	2.18	**H33**	3.81	**H52'**	2.10
H17	4.10	**H34**	1.79	**H53**	4.42
H18	2.26	**H34'**	2.16	**H54**	5.19
H18'	2.80	**H35**	4.11	**H55**	4.41
19=CH_2	4.93	**H36**	4.10	**H55'**	4.41
19'=CH_2	5.00	**H37**			
H20	4.38	**H37'**	2.35		

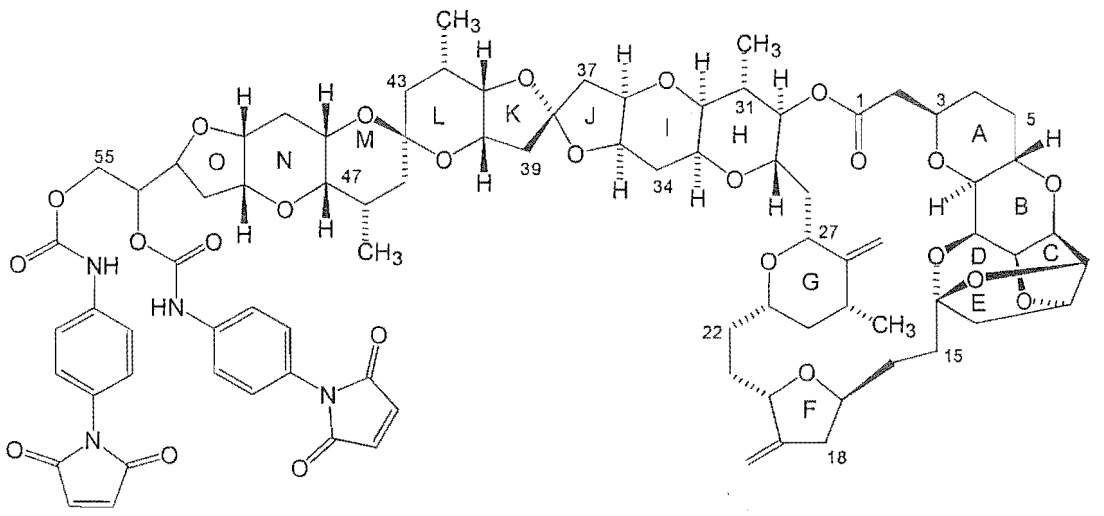
[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Figure 4.3.2 Homohalichondrin B PMPI Hapten – Important 2D-TOCSY Correlations



The spectral window used for the 2D experiments was limited to the halichondrin region of the spectrum. Consequently, data for the linker portion of **4.8** are not available. These data confirmed the connectivity and chemical shift of the A-N rings were identical to those of the parent **1.25**. A TOCSY correlation between the H51 resonance (δ_H 4.04) and a resonance at δ_H 4.42 allowed the assignment of H53. A correlation between the H53 proton resonance and a proton resonating at δ_H 5.19 allowed the assignment of H54. The H55 proton resonance was assigned from the observation of a TOCSY correlation between a doublet resonance at δ_H 4.41 and the H54 resonance. No mass spectrometry data could be obtained for **4.8**. HRFABMS data were obtained for the related **4.6** (Section 4.2.3) which has two PMSI (**4.1**) linkers to attached to **1.25**. The product **4.6** is similar in polarity to **4.8**. The relative polarity of **4.8**, the pair of maleimide resonances observed in the ^{1}H NMR spectrum of **4.8**, and the HRFABMS result for the related **4.6** are all consistent with formation of the disubstituted product **4.8**.



homohalichondrin B PMPI hapten (**4.8**)

4.3.2.2 Characterisation of **4.9**

The molecular formula of the major component **4.9** was determined to be $C_{72}H_{92}O_{22}N_2$ by HRFABMS, indicating attachment of a single linker **4.2**. The major differences in

the ^{1}H NMR spectrum of **4.9** (Figure 4.3.3) compared to that of **1.25** were the appearance of extra resonances at δ_H 4.2 and δ_H 3.8, and the absence of the characteristic H55/H55' resonance at δ_H 3.69. 2D-TOCSY (mixing time 80 ms) and HSMQC experiments were run to quickly obtain a maximum amount of spectral data. The partially assigned ^{1}H NMR and ^{13}C NMR data are collated in Table 4.3.2 and Table 4.3.3, and important 2D correlations are shown in Figure 4.3.4.

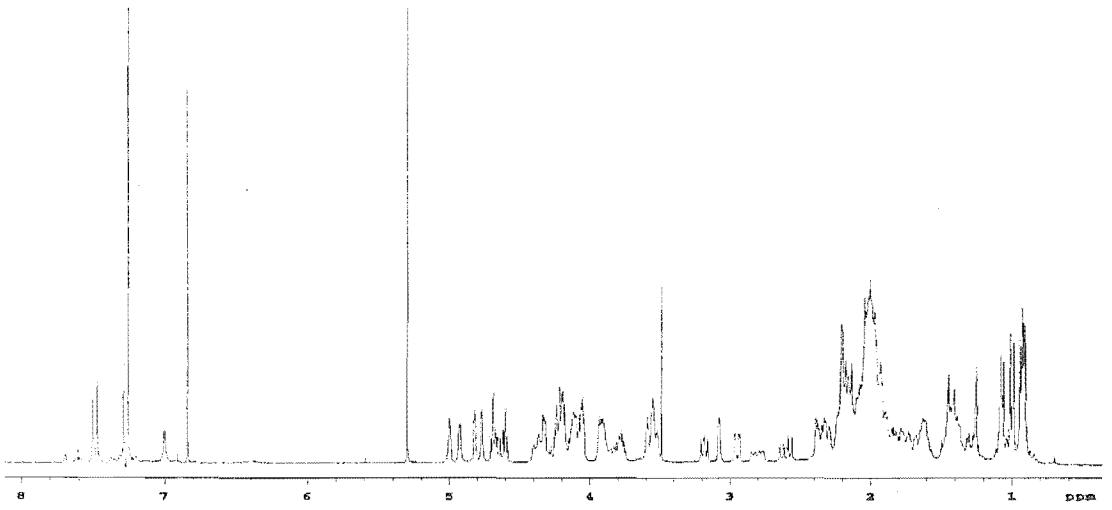


Figure 4.3.3 ^{1}H NMR Spectrum of Homohalichondrin B PMPI Hapten (**4.9**)

Table 4.3.2 ^{1}H NMR Data for Homohalichondrin B PMPI Hapten (**4.9**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.35	**H22**	1.61	**H42**	2.34
H2'	2.60	**H22'**	1.61	**CH_3-42**	0.93
H3	3.88	**H23**	3.54	**H43**	1.28
H4	1.38	**H24**	1.04	**H43'**	1.48
H4'	1.74	**H24'**	1.69	**H45**	1.37
H5		**H25**	2.21	**H45'**	1.49
H5'	2.09	**CH_3-25**	1.07	**H46**	2.17
H6	4.36	**26=CH_2**	4.77	**CH_3-46**	0.92
H7	2.95	**26'=CH_2**	4.82	**H47**	3.08
H8	4.32	**H27**	3.53	**H48**	3.54
H9	4.05	**H28**		**H49**	1.80
H10	4.20	**H28'**		**H49'**	2.18
H11	4.60	**H29**	4.19	**H50**	3.90
H12	4.69	**H30**	4.66	**H51**	4.05
H13	1.94	**H31**	2.03	**H52**	
H13'	2.15	**CH_3-31**	1.00	**H52'**	
H15	1.61	**H32**	3.18	**H53**	4.21
H15'	2.17	**H33**	3.81	**H54**	3.76[c]
H16	1.42	**H34**	1.79	**H55**	4.23[c]
H16'	2.17	**H34'**	2.15	**H55'**	4.23[c]
H17	4.10	**H35**	4.11	**H2'**	6.84
H18	2.26	**H36**	4.11	**H3'**	6.84
H18'	2.80	**H37**	1.88	**H6'**	7.27
19=CH_2	4.92	**H37'**	2.35	**H7'**	7.49
19'=CH_2	5.00	**H39**	2.20	**H9'**	7.49
H20	4.38	**H39'**	2.20	**H10'**	7.27
H21	1.89	**H40**	3.93		
H21'	1.89	**H41**	3.59		

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

[c] Assignment of H54 and H55/55' tentative – may be interchanged

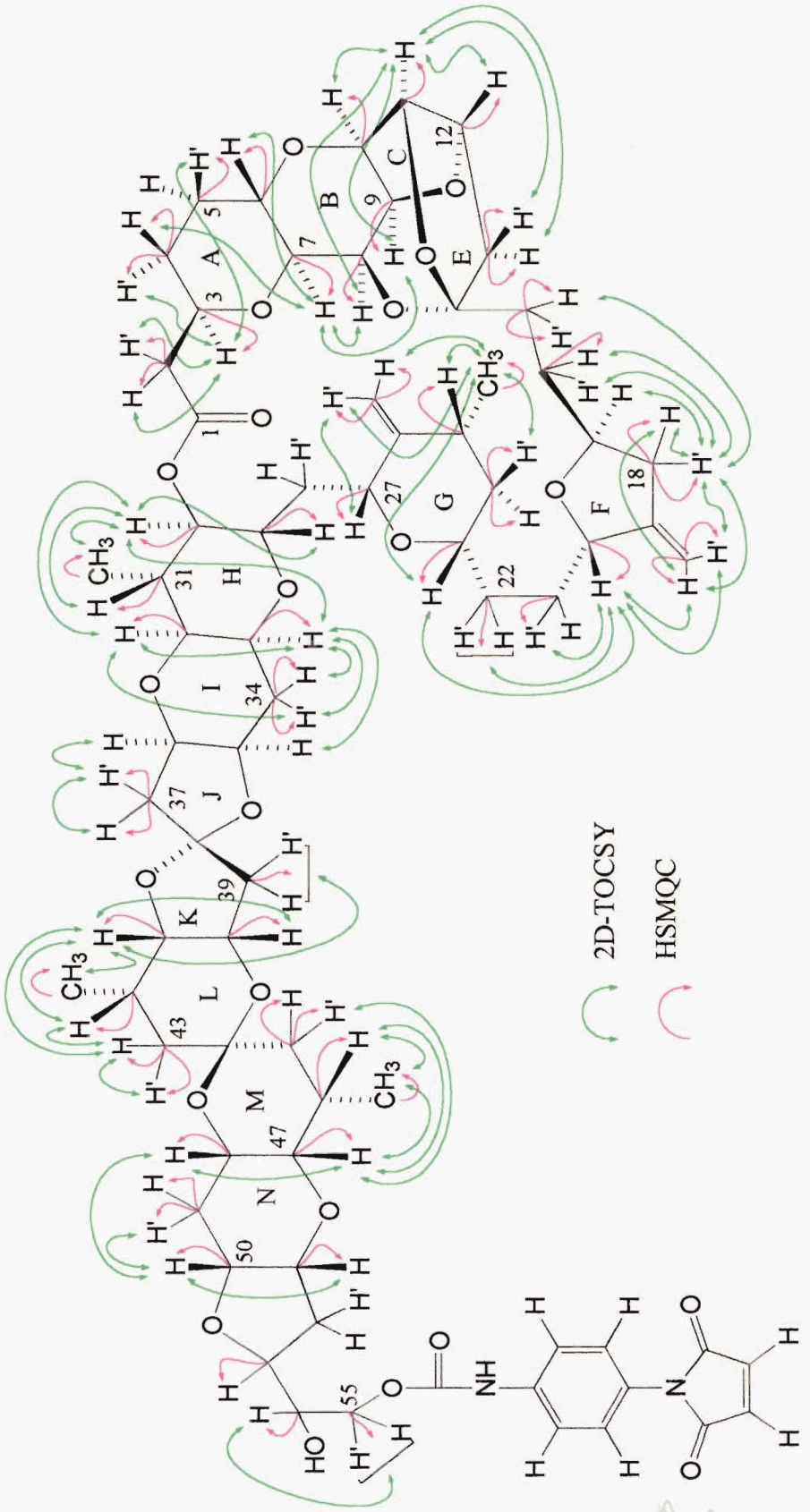
Table 4.3.3 ^{13}C NMR Data for Homohalichondrin B PMPI Hapten (**4.9**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1		C21	29.2	C39	42.4
C2	40.2	C22	31.8	C40	70.7
C3	73.6	C23	74.9	C41	79.5
C4	30.5	C24	43.2	C-CH_3-42	25.4
C5	29.8	C-CH_3-25	35.8	C-CH_3-42	17.4
C6	68.2	C-CH_3-25	17.8	C43	36.9
C7	77.6	26C=CH_2		C44	
C8	74.3	26C=CH_2	103.9	C45	36.9
C9	73.9	C27	73.6	C-CH_3-46	28.7
C10	76.6	C28		C-CH_3-46	16.9
C11	81.9	C29	71.2	C47	73.0
C12	80.8	C30	77.1	C48	63.5
C13	48.2	C-CH_3-31	36.6	C49	31.0
C14		C-CH_3-31	14.8	C50	74.6
C15	34.2	C32	77.6	C51	76.6
C16	28.0	C33	66.5	C52	
C17		C34	28.9	C53	77.8
C18	38.6	C35		C54	67.3[b]
19C=CH_2	151.3	C36		C55	71.7[b]
19C=CH_2	104.3	C37	43.2		
C20	75.2	C38			

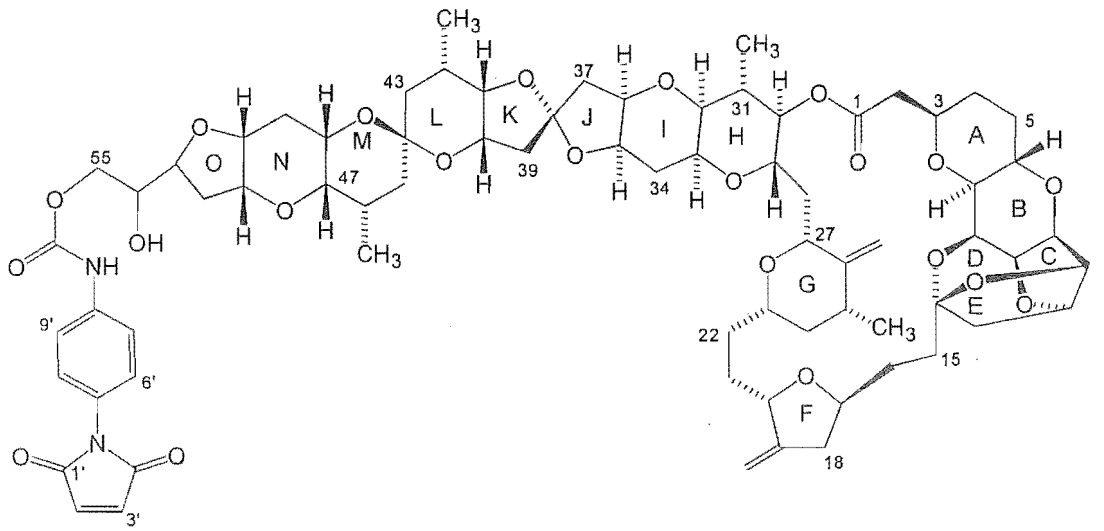
[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from an HSMQC spectrum at 300 MHz.

[b] Assignments tentative – may be interchanged

Figure 4.3.4 Homohalichondrin B Hapten – Important NMR Correlations



The carbons of the linker portion of **4.9** were not assigned owing to the narrowed window (δ_H 5.2-0.8) used for the 2D NMR experiments. The majority of the methine carbons of **4.9** were assigned by direct comparison of the HSMQC data to those of homohalichondrin B (**1.25**). Three carbon resonances remained unassigned: δ_C 71.7, δ_C 77.8 and δ_C 67.3. These carbon resonances were correlated to protons resonating at δ_H 3.76, δ_H 4.21 and δ_H 4.23 respectively. It was not possible to assign any of the H53, H54 or H55/H55' protons from the available spectral data. No TOCSY correlation was seen between H50 and any of these resonances. A correlation between the H9 resonance (δ_H 4.05) and the resonance of H10 (δ_H 4.20) overlapped with any possible correlation between the H51 resonance (δ_H 4.05) and either of the two protons resonating at *ca* δ_H 4.2. The carbon resonating at δ_C 77.8 was assigned to C53 on the basis of chemical shift arguments. An attempt to assign the remaining two carbon/proton resonances was made by comparison of chemical shift differences. The change in chemical shift upon converting an alcohol to a carbamate was compared for the α-methylene protons of **1.25/4.9** and 2,3-di-*O*-methyl-*L*-ascorbic acid and its PMPI reaction product. However the differences observed were not consistent with either assignment of C54/H54 and C55/H55/55'.

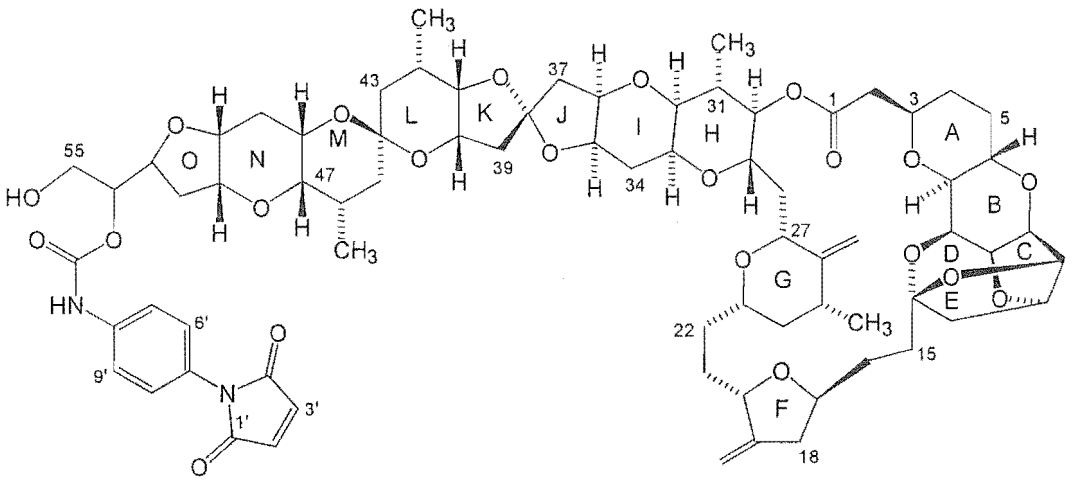


homohalichondrin B PMPI hapten (**4.9**)

The available spectral data confirmed the structure of **4.9** to be the result of reaction at the primary C55 hydroxy group.

4.3.2.3 Characterisation of **4.10**

The observation of a single maleimide proton (δ_H 6.84) in the ^{1}H NMR spectrum of **4.10** was indicative of the addition of a single PMPI (**4.2**) linker to **1.25**. The ^{1}H NMR spectrum of **4.10** contained a distinctive new multiplet resonating at δ_H 4.52. Other new resonances were observed at δ_H 3.90 and under the 19=CH_2 resonance (δ_H 4.92). A 2D-TOCSY (mixing time 80 ms) experiment and an HSMQC experiment were run to obtain connectivity information. The partially assigned ^{1}H NMR and ^{13}C NMR data are collated in Table 4.3.4 and Table 4.3.5. The important 2D NMR correlations are shown in Figure 4.3.5.



homohalichondrin B PMPI hapten (**4.10**)

Table 4.3.4 ^{1}H NMR Data for Homohalichondrin B PMPI Hapten (**4.10**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H21**		**H39**	2.20
H2'	2.60	**H21'**		**H39'**	2.20
H3	3.87	**H22**	1.60	**H40**	3.93
H4		**H22'**	1.60	**H41**	3.62
H4'	1.74	**H23**	3.53	**H42**	2.33
H5		**H24**		**CH_3-42**	0.96
H5'	2.10	**H24'**	1.69	**H43**	1.28
H6	4.35	**H25**	2.21	**H43'**	1.49
H7	2.95	**CH_3-25**	1.07	**H45**	
H8	4.32	**26=CH_2**	4.77	**H45'**	1.44
H9	4.05	**26'=CH_2**	4.82	**H46**	2.16
H10	4.18	**H27**	3.54	**CH_3-46**	0.92
H11	4.60	**H28**		**H47**	3.08
H12	4.69	**H28'**		**H48**	3.54
H13	1.94	**H29**	4.19	**H49**	
H13'	2.15	**H30**	4.66	**H49'**	
H15		**H31**	2.02	**H50**	3.93
H15'		**CH_3-31**	1.00	**H51**	4.04
H16		**H32**	3.19	**H52**	2.06
H16'	2.16	**H33**	3.81	**H52'**	2.06
H17	4.09	**H34**	1.78	**H53**	4.52
H18	2.26	**H34'**	2.16	**H54**	4.92
H18'	2.81	**H35**	4.11	**H55**	3.90
19=CH_2	4.92	**H36**	4.10	**H55'**	3.90
19'=CH_2	5.00	**H37**			
H20	4.38	**H37'**	2.35		

[a] The symbol ' represents the less shielded proton of a geminal pair.

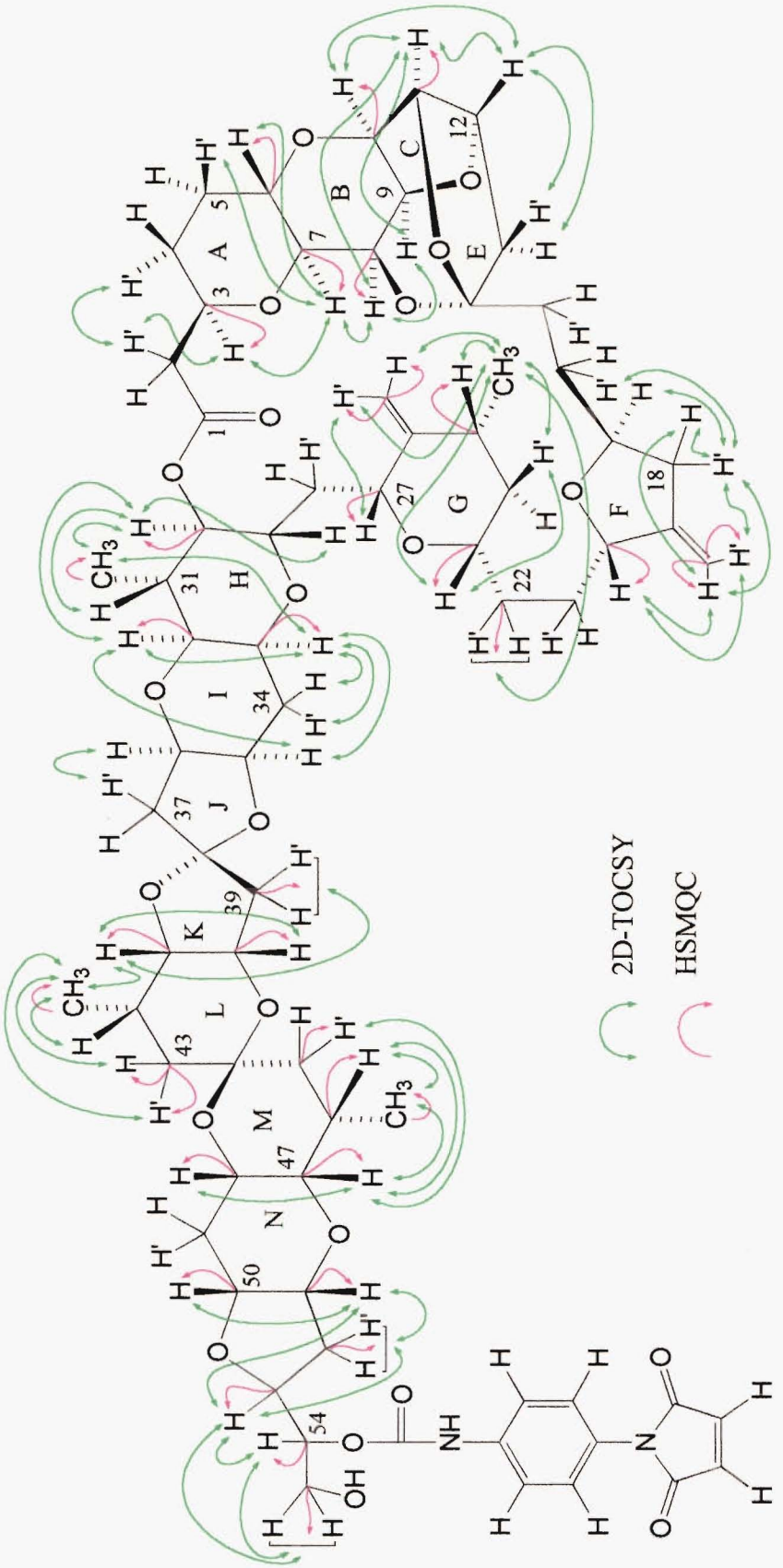
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 4.3.5 ^{13}C NMR Data for Homohalichondrin B PMPI Hapten (**4.10**)

Carbon	δ ppm[a]	**Carbon**	δ ppm[a]	**Carbon**	δ ppm[a]
C1		**C21**		**C39**	42.4
C2		**C22**	31.7	**C40**	70.8
C3	73.4	**C23**	74.9	**C41**	79.3
C4		**C24**		**C-CH_3-42**	
C5		**C-CH_3-25**		**C-CH_3-42**	17.4
C6	68.0	**C-CH_3-25**	17.8	**C43**	37.1
C7	77.3	**26C=CH_2**		**C44**	
C8	74.5	**26C=CH_2**	103.9	**C45**	37.1
C9		**C27**	73.4	**C-CH_3-46**	28.4
C10	76.4	**C28**		**C-CH_3-46**	17.0
C11	81.8	**C29**		**C47**	72.9
C12		**C30**	77.0	**C48**	63.5
C13		**C-CH_3-31**		**C49**	
C14		**C-CH_3-31**	14.7	**C50**	74.0
C15		**C32**	77.5	**C51**	75.5
C16		**C33**	66.6	**C52**	36.6
C17		**C34**		**C53**	
C18		**C35**		**C54**	74.8
19C=CH_2		**C36**		**C55**	64.0
19C=CH_2	104.3	**C37**			
C20	75.5	**C38**			

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from an HSMQC spectrum at 300 MHz.

Figure 4.3.5 Homohalichondrin B PMPI Hapten – Important NMR Correlations



The spectral window used for the 2D experiments was limited to the halichondrin region of the spectrum. Consequently data for the linker portion of **4.10** are not available. A correlation between the H51 resonance (δ_H 4.04) and δ_H 4.52 allowed the assignment of H53. The H52/H52' methylene resonance was assigned from the observation of a TOCSY correlation from both the H51 resonance and the H53 resonance to δ_H 2.06. The H53 resonance was also correlated to δ_H 4.92 and δ_H 3.90. The upfield (δ_H 3.90) proton resonance was correlated to a carbon resonating at δ_C 64.0 allowing the assignment of H55. The proton resonating at δ_H 4.92 was thus assigned as H54. An HSMQC correlation was observed from this proton resonance to δ_C 74.8. The difference in chemical shift for C54 in **4.10** relative to the parent **1.25** (δ_C 2.8 downfield) supported the assignment of **4.10** as being the secondary hydroxy-linked hapten.

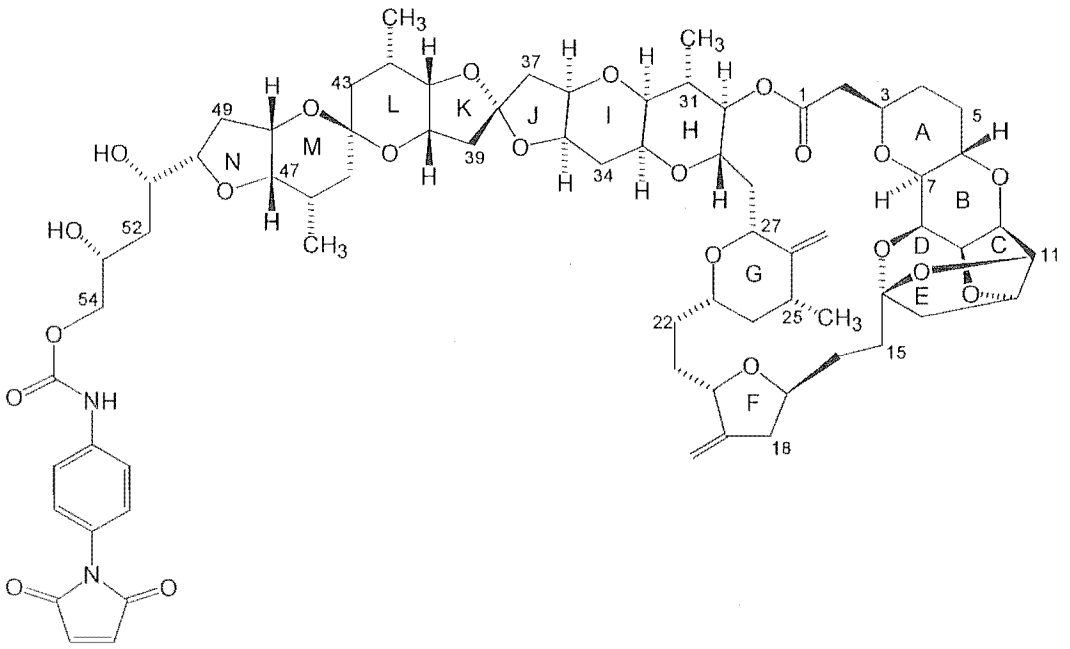
4.3.3 Halichondrin B and PMPI

Halichondrin B (**1.8**, 1.5 mg) was treated with an equimolar amount of **4.2** in $CDCl_3$. No further changes were observed in the ^{1}H NMR spectrum of the product mixture after 72 hours. The product mixture was analysed by reverse phase (C18) HPLC, using 60% CH_3CN/H_2O as the mobile phase. The mixture contained two major components (**1.8**, 229 s, **4.11**, 374 s) and several minor components of varying polarity. The relative polarities of the components were indicative of the formation of mono-, bis- and tri-substituted halichondrin B derivatives. Integration of the product peaks showed the major component was *ca* 80% of the product mixture. This would be the lower bound of the estimate as the bis and trisubstituted components have a more intense UV chromophore. Semipreparative reverse phase (C18) HPLC was used to separate the mixture into six components.

4.3.3.1 Characterisation

The most polar fraction was identified as the starting material **1.8** by comparison of its ^{1}H NMR spectrum with that of an authentic sample of **1.8**.

The major component **4.11** was found to have a molecular formula of $C_{71}H_{92}O_{22}N_2$ by HRFABMS, consistent with the addition of a single **4.2** linker. In order to determine the position of the linker, a 1D TOCSY experiment was run on both a standard sample of **1.8** (mixing time 100 ms, Figure 4.3.6) and **4.11** (mixing time 80 ms). The H51 proton (δ_H 3.80) was irradiated to probe the spin system around to H54. The H51 resonance overlapped with the H33 resonance (also δ_H 3.80) hence the need to fully assign the 1D-TOCSY spectrum of **1.8**. The H54/H54' pair of doublet of doublets was observed to shift from δ_H 3.54 and δ_H 3.61 to *ca* δ_H 4.10 and δ_H 4.20. This change in chemical shift is consistent with the addition of a PMPI (**4.2**) linker to the C54 hydroxy group. Attachment of the **4.2** linker at the C53 hydroxy group would be expected to cause a shift downfield by at least δ_H 0.6 of the H53 resonance. This change in chemical shift was not observed in the 1D TOCSY spectrum of **4.11**.



halichondrin B PMPI hapten (**4.11**)

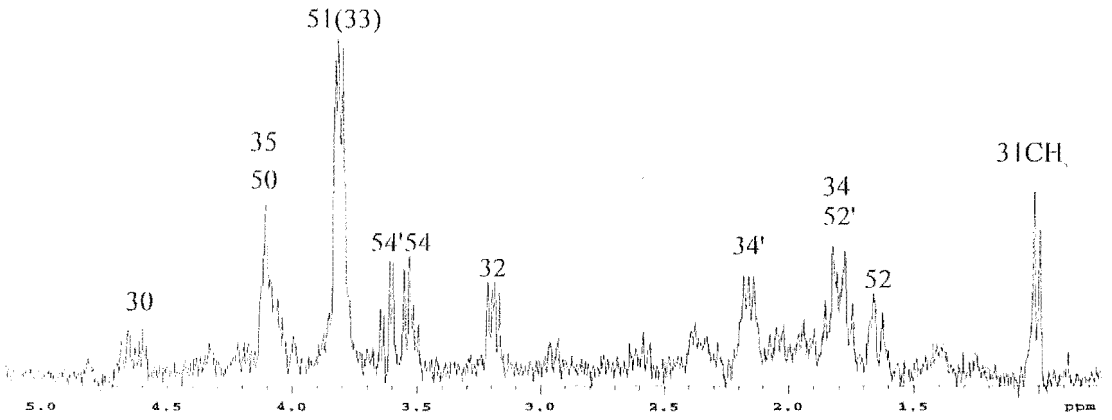
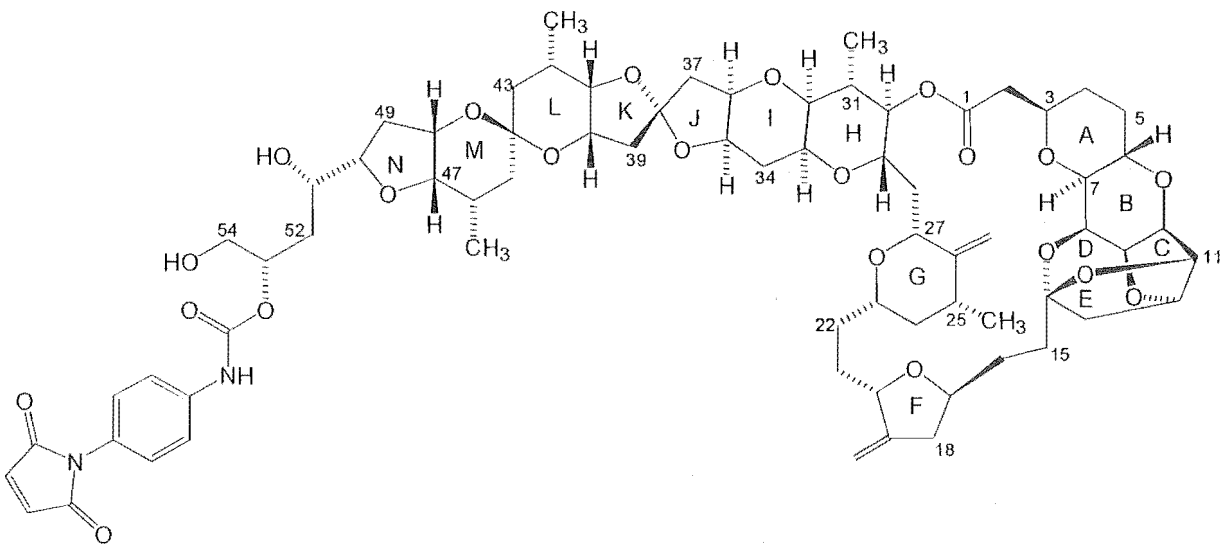


Figure 4.3.6 1D-TOCSY Irradiating H51 of **1.8**

A minor component **4.12** was found to be isobaric to **4.11** by HRFABMS. This is consistent with the order of elution from C18. Compound **4.12** was the most polar of the products isolated. The single **4.2** linker could be attached at the C51 hydroxy group or the C53 hydroxy group. An unmasked diol (linker attached at the C51 hydroxy group) would possibly give rise to the more polar compound. A lack of material prevented the use of NMR techniques to confirm this supposition.



halichondrin B PMPI hapten (**4.12**)

The three remaining fractions were all of a low mass (< 0.1 mg) which precluded the use of ^{1}H NMR spectroscopy. These samples were submitted for MS analysis but no

The three remaining fractions were all of a low mass (< 0.1 mg) which precluded the use of ^{1}H NMR spectroscopy. These samples were submitted for MS analysis but no useful results were obtained. The more polar of these fractions eluted from C18 in 346 s, after **1.8** (229 s) and **4.12** (346 s) but before **4.11** (374 s). The relative polarity of this component is consistent with the addition of a single **4.2** linker to the C53 (or C51) hydroxy group. A combination of three compounds, eluting from 467 s to 523 s, was collected as a single fraction. It is possible that this fraction contains a mixture of disubstituted **1.8**. The remaining fraction was relatively less polar, eluting at 692 s. This fraction may be the result of linker attachment at all three hydroxy groups of **1.8**.

4.4 Norhomohalichondrin B Aldehyde

4.4.1 Introduction

Modification of the terminal diol of homohalichondrin B (**1.25**) was desirable, as this would create another option for linker attachment. Sodium periodate was chosen as the reagent for diol cleavage because it had been used previously to successfully treat olefin modified halichondrins.[95]

4.4.2 Diol Cleavage

A small quantity of homohalichondrin B (**1.25**) was stirred with an aqueous solution of sodium periodate at room temperature overnight. A doublet resonance at δ_H 9.69 in the ^{1}H NMR spectrum of the product indicated formation of the aldehyde **4.13**.

The reaction was repeated on a larger scale to provide enough material for thorough structure elucidation.

4.4.3 Structural Elucidation

The molecular formula of **4.13** was determined as $C_{60}H_{82}O_{18}$ by HRFABMS. The ^1H NMR spectrum of **4.13** is displayed in Figure 4.4.1. Irradiation of the aldehyde resonance (δ_H 9.69) was used to locate the decoupled H53 proton resonance at δ_H 4.51.

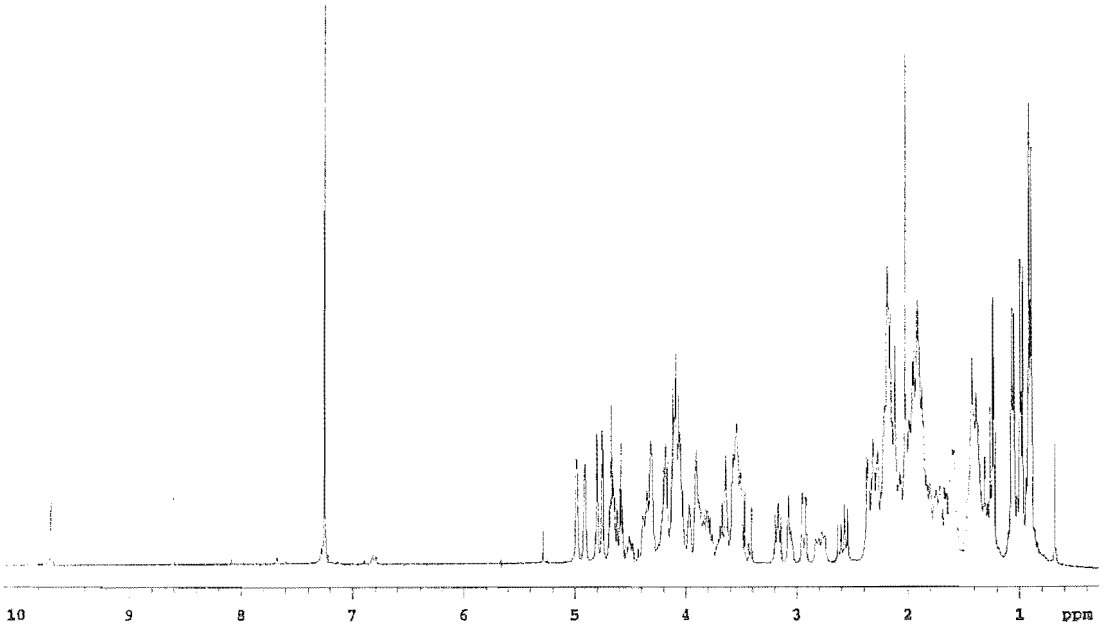


Figure 4.4.1 ^1H NMR Spectrum of Norhomohalichondrin B Aldehyde (**4.13**)

A full array of NMR experiments were performed on **4.13** *viz* COSY, 2D-TOCSY (mixing time 80 ms), HSMQC, HMBC, NOE and APT (Figure 4.4.2) experiments. These data enabled the complete assignment of the ^1H NMR and ^{13}C NMR resonances of **4.13** to be achieved. The ^1H and ^{13}C spectral data are listed in Table 4.4.1 and Table 4.4.2 respectively, and the important correlations from the 2D experiments are shown in Figure 4.4.3.

Table 4.4.1 ^{1}H NMR Data for Norhomohalichondrin B Aldehyde (**4.13**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.33	**H20**	4.37	**H37**	1.89
H2'	2.58	**H21**	1.38	**H37'**	2.34
H3	3.87	**H21'**	1.88	**H39**	2.21
H4	1.37	**H22**	1.60	**H39'**	2.21
H4'	1.72	**H22'**	1.60	**H40**	3.92
H5	1.37	**H23**	3.53	**H41**	3.58
H5'	2.07	**H24**	1.03	**H42**	2.32
H6	4.35	**H24'**	1.69	**CH_3-42**	0.91
H7	2.94	**H25**	2.19	**H43**	1.27
H8	4.31	**CH_3-25**	1.06	**H43'**	1.41
H9	4.04	**26=CH_2**	4.75	**H45**	1.42
H10	4.18	**26'=CH_2**	4.80	**H45'**	1.42
H11	4.59	**H27**	3.52	**H46**	2.17
H12	4.68	**H28**	1.93	**CH_3-46**	0.91
H13	1.94	**H28'**	2.03	**H47**	3.08
H13'	2.14	**H29**	4.19	**H48**	3.55
H15	1.60	**H30**	4.65	**H49**	1.84
H15'	2.17	**H31**	2.01	**H49'**	2.24
H16	1.42	**CH_3-31**	0.99	**H50**	3.97
H16'	2.16	**H32**	3.17	**H51**	4.06
H17	4.09	**H33**	3.80	**H52**	1.97
H18	2.25	**H34**	1.80	**H52'**	2.27
H18'	2.79	**H34'**	2.14	**H53**	4.51
19=CH_2	4.91	**H35**	4.11	**H54**	9.69
19'=CH_2	4.98	**H36**	4.11		

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 4.4.2 ^{13}C NMR Data for Norhomohalichondrin B Aldehyde (**4.13**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.0	**C20**	75.3	**C37**	43.5
C2	40.5	**C21**	29.5	**C38**	112.3
C3	73.7	**C22**	32.1	**C39**	42.6
C4	30.8	**C23**	74.8	**C40**	70.8
C5	30.1	**C24**	43.4	**C41**	79.3
C6	68.2	**$\underline{C}$-CH_3-25**	36.0	**$\underline{C}$-CH_3-42**	25.8
C7	77.7	**C-$\underline{C}H_3$-25**	18.1	**C-$\underline{C}H_3$-42**	17.7
C8	74.3	**26$\underline{C}$=CH_2**	151.6	**C43**	37.0
C9	73.9	**26C=$\underline{C}H_2$**	104.1	**C44**	96.7
C10	76.5	**C27**	73.5	**C45**	37.0
C11	82.1	**C28**	37.0	**$\underline{C}$-CH_3-46**	28.8
C12	81.1	**C29**	71.2	**C-$\underline{C}H_3$-46**	17.1
C13	48.4	**C30**	77.0	**C47**	73.3
C14	110.0	**$\underline{C}$-CH_3-31**	36.6	**C48**	63.3
C15	34.5	**C-$\underline{C}H_3$-31**	15.1	**C49**	31.1
C16	28.2	**C32**	77.6	**C50**	75.8
C17	75.4	**C33**	66.6	**C51**	76.0
C18	38.8	**C34**	29.1	**C52**	36.2
19$\underline{C}$=CH_2	151.4	**C35**	75.0	**C53**	82.0
19C=$\underline{C}H_2$	104.4	**C36**	76.3	**C54**	202.7

[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

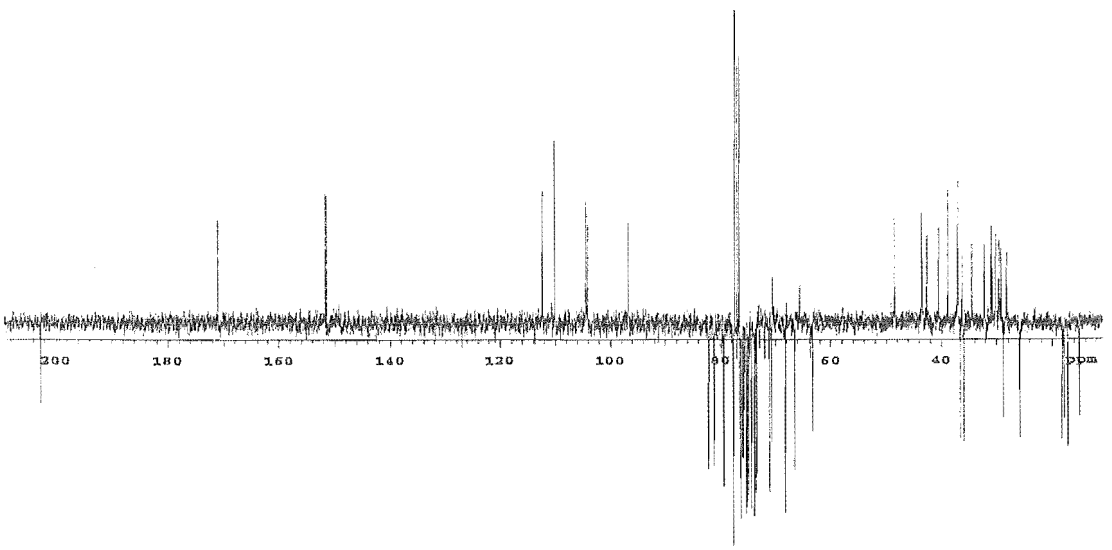
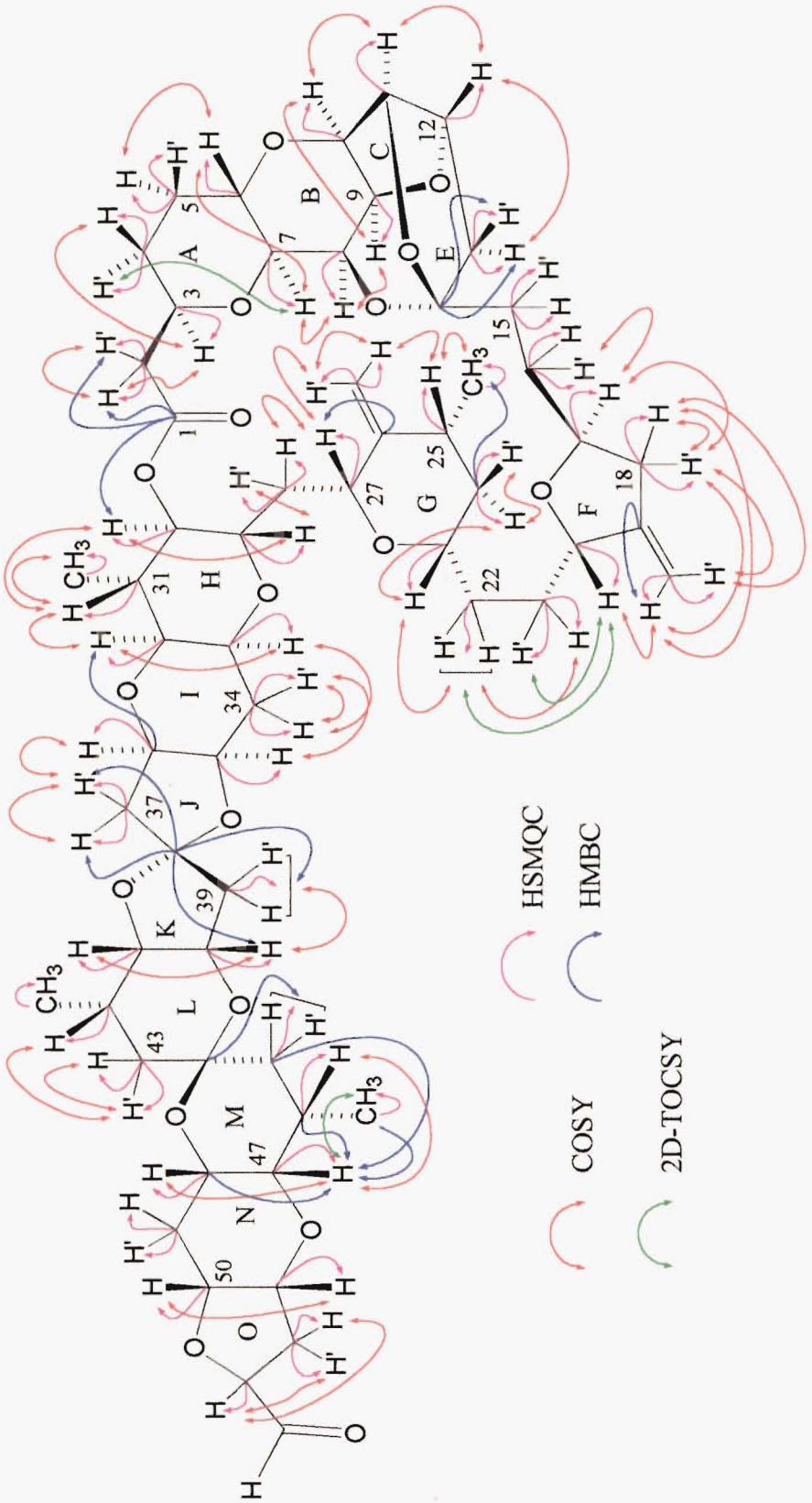
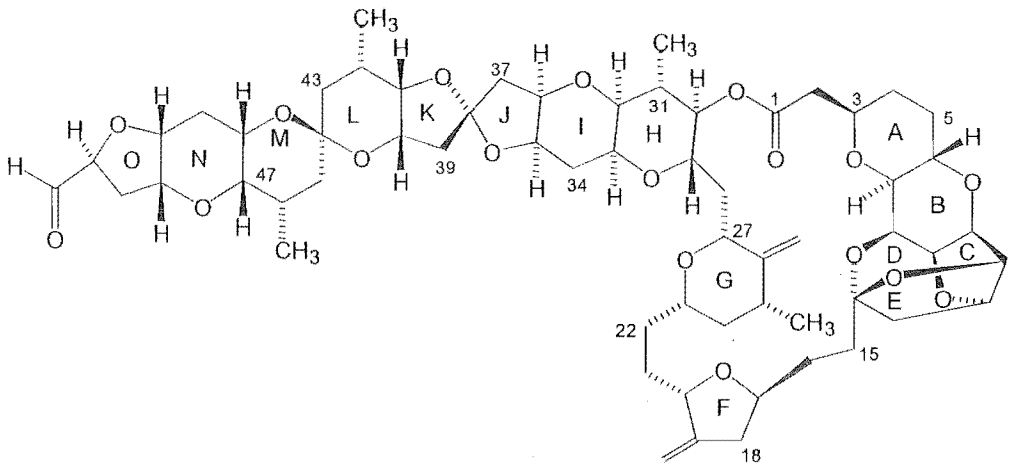


Figure 4.4.2 APT Spectrum of Norhomohalichondrin B Aldehyde (**4.13**)

Figure 4.4.3 Norhomohalichondrin B Aldehyde – Important NMR Correlations



These data confirmed the connectivities and NMR shifts of the A-M rings were identical to those of homohalichondrin B (**1.25**). Correlations in the COSY spectrum from the previously assigned H53 resonance (δ_H 4.51) to resonances at δ_H 1.97 and δ_H 2.27 allowed the assignment of H52 and H52'. A correlation in the 2D-TOCSY spectrum between the H53 resonance and δ_H 4.06 allowed the assignment of H51. The H51 resonance was correlated to a resonance at δ_H 3.97, enabling the assignment of H50. A COSY correlation between the H48 resonance (δ_H 3.55) and δ_H 1.84 allowed the assignment of the H49 resonance. The remaining H49' resonance was assigned from the observation of a COSY correlation between δ_H 1.84 (H49) and δ_H 2.24.



norhomohalichondrin B aldehyde (**4.13**)

4.4.4 Stereochemistry

4.4.4.1 Introduction

The relative stereochemistry of H53 in homohalichondrin B (**1.25**) has an element of uncertainty associated with the assignment. The formation of **4.13** provided an opportunity to examine and assign this relative stereochemistry as the H53 proton was now observed as an isolated resonance in the ^{1}H NMR spectrum of **4.13**. Unfortunately, no NOE enhancements were observed between protons across the O ring. Modelling of this five membered ring showed that the lack of NOE enhancements was to be expected.

4.4.4.2 Proton Decoupling Experiment

Irradiation of the H54 aldehyde resonance in a ^1H decoupling experiment allowed the coupling between H53 and the H52/H52' methylene pair to be observed. The observed coupling constants ($^3J_{HH}$) were 6.46 Hz between H52 and H53, and 10.26 Hz between H52' and H53.

4.4.4.3 Molecular Modelling

The M, N and O rings of **4.13** were generated in ChemDraw Pro, incorporating the known stereochemistry and the *cis* and *trans* options for H53. The structures were transferred *via* Chem3D Pro to MacroModel. The energies of the two structures were minimised using an MM2* forcefield, incorporating solvent effects ($CHCl_3$). Vicinal coupling constants were collected in MacroModel for the minimised structures and compared to the experimentally obtained $^3J_{HH}$ coupling constants. The coupling constants derived from the modelled structures (Figure 4.4.4, 6.2 Hz, 10.7 Hz) most closely resembled the experimental values for the *trans* configuration. The $^3J_{HH}$ coupling constants extracted from MacroModel for the *cis* configuration (1.4 Hz, 8.2 Hz) were not consistent with the experimentally observed coupling constants for H53.

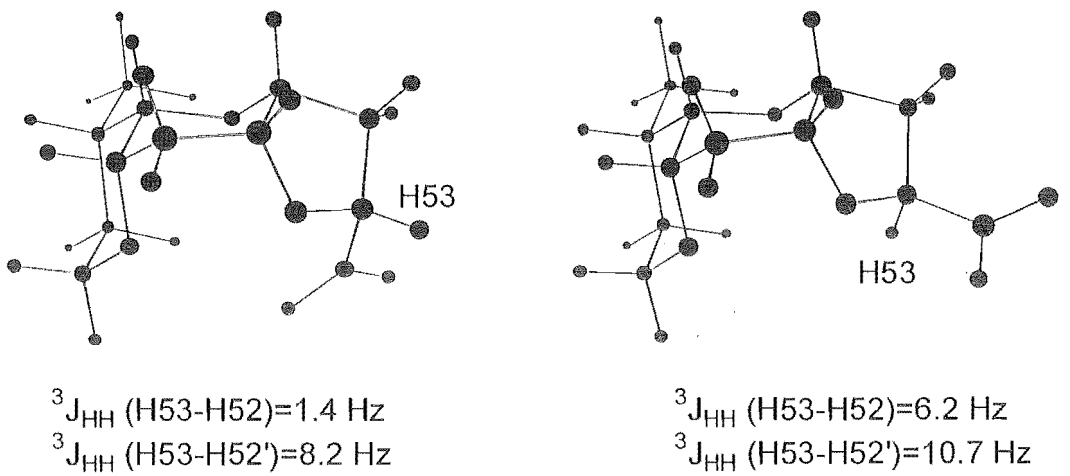


Figure 4.4.4 Models of the Two Possible Configurations of the C53 Stereocentre

A recent paper has presented views on the stereochemistry of the C53 stereocentre in conflict with that originally proposed. Uemura reported that the relative stereochemistry of homohalichondrin B had been *deduced from spectral data and biogenic considerations* (Figure 4.4.5).[96]

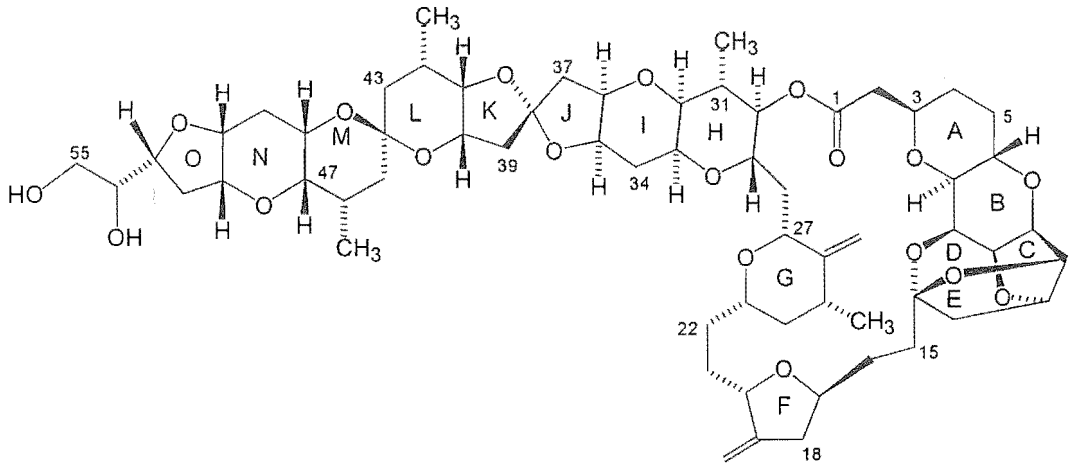
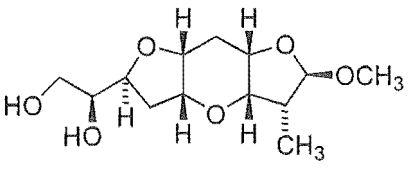


Figure 4.4.5 Stereochemistry of Homohalichondrin B Reported by Uemura

As part of the synthesis of the left half of the homohalichondrins, Kishi constructed all possible stereoisomers with respect to the C53 and C54 positions of homohalichondrin B (**1.25**).[97] Only diastereomer **4.14** exhibited ^{1}H NMR data close to that of the data reported for the natural product. It should be noted that Uemura's proposed stereochemistry for C53 is opposite to that found by Kishi.



Kishi's diastereomer (**4.14**)

The stereochemistry of C53 determined in this thesis by comparison of experimental and modelled coupling constants agrees with that reported by Kishi for the left hand portion of homohalichondrin B.

4.4.5 Biological Activity

The biological activity of norhomohalichondrin B aldehyde (**4.13**) was evaluated in the *in vitro* P388 murine leukemia assay, and in the NCI's primary screening system. Included with the results in Table 4.4.3 below are those of the parent halichondrin **1.25** and halichondrin B (**1.8**). The GI_{50}-centred mean graph profile of halichondrin B (**1.8**) was used as the "seed" for the calculation of COMPARE correlation coefficients.

Table 4.4.3 *In Vitro* Cytotoxicities of Selected Halichondrins

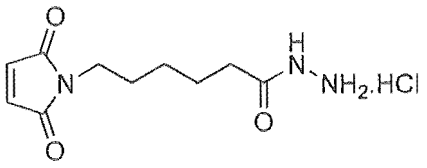
Compound	**P388 IC_{50} (ng/mL)**	**NCI GI_{50} ($\times 10^{-10}$ M)**	**COMPARE Correlation**
halichondrin B (1.8)	0.78	1.38	1.00
homohalichondrin B (1.25)	0.22	3.16	0.95
norhomohalichondrin B aldehyde (4.13)	0.33	3.02	0.98

The derivative **4.13** possesses activity comparable to that of the parent halichondrin **1.25**. The mechanism of action of **4.13** is also very closely correlated to that of the "seed" compound, **1.8.** This result is not unexpected, as it has been observed previously[95] that alteration of the terminal diol of **1.25** has little effect on the biological activity.

4.5 EMCH Linker-derived Haptens

4.5.1 Introduction

E-Maleimidocaproic acid hydrazide (EMCH, **4.15**) is a carbonyl-reactive linker. It consists of a sulfhydryl reactive maleimide for subsequent conjugation of the hapten to a protein, an amino caproic acid spacer unit, and the nucleophilic hydrazide.



EMCH (**4.15**)

4.5.2 Norhomohalichondrin B Aldehyde and EMCH

The reaction conditions for coupling the aldehyde **4.13** to EMCH (**4.15**) were first established using butyraldehyde in place of **4.13**.

Norhomohalichondrin B aldehyde (**4.13**) was treated with one equivalent of **4.15** and two equivalents of sodium acetate overnight. The absence of the characteristic **4.13** H53 and H54 resonances in the ^{1}H NMR spectrum (Figure 4.5.1) of the freeze dried reaction mixture indicated reaction of the aldehyde **4.13** was complete.

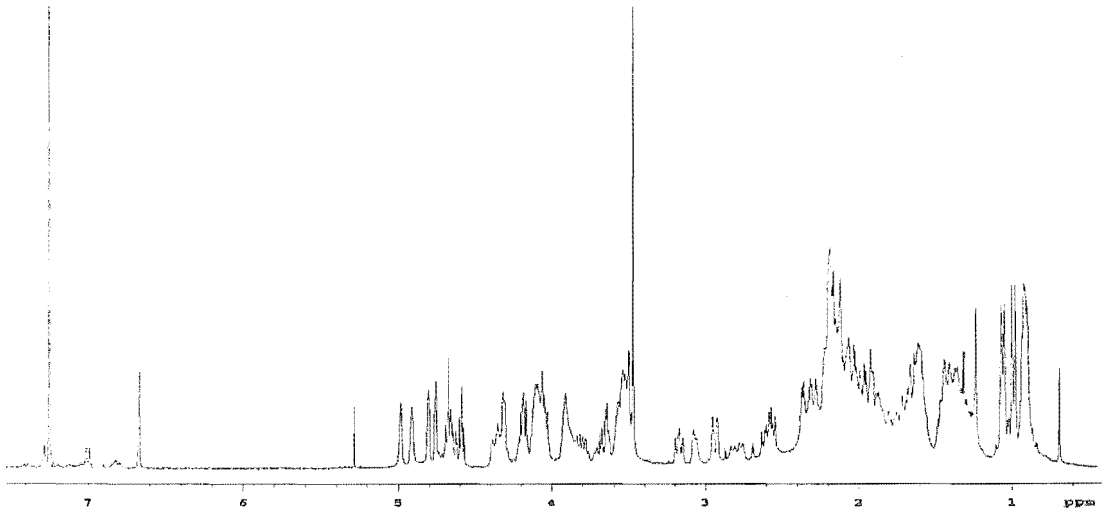
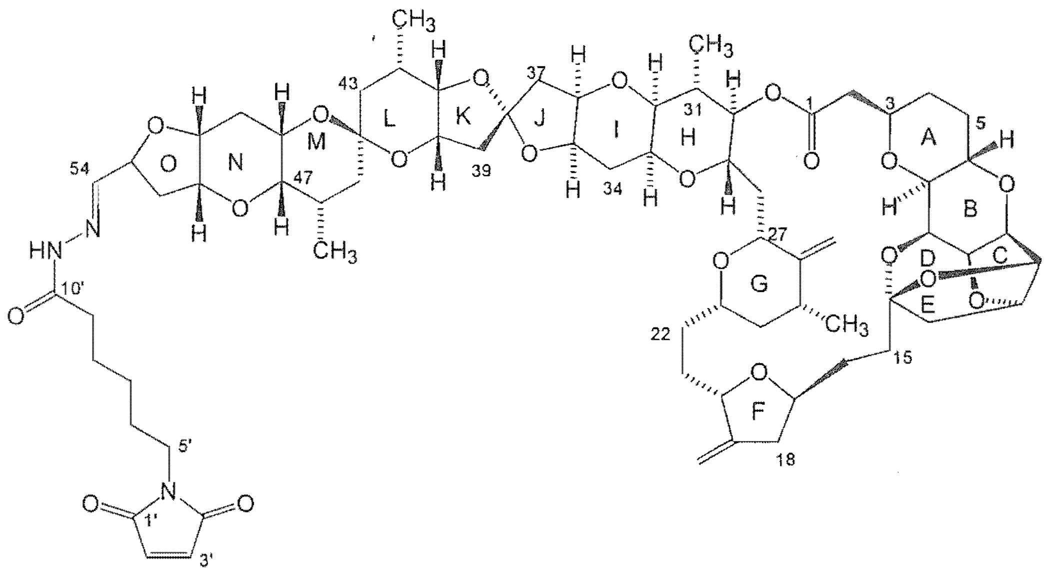


Figure 4.5.1 ^{1}H NMR Spectrum of EMCH Hapten (**4.16**)

4.5.2.1 Characterisation of **4.16**

The molecular formula of **4.16** was determined as $C_{70}H_{95}O_{20}N_3$ by HRFABMS. 2D-TOCSY (mixing time 80 ms), COSY and HSMQC NMR experiments were performed on **4.16**. These spectral data were used to partially assign the ^{1}H and ^{13}C resonances of

4.16. These assignments are listed in Table 4.5.1 and Table 4.5.2 respectively. The important correlations from the 2D experiments are shown in Figure 4.5.2.



EMCH hapten (**4.16**)

Table 4.5.1 ^{1}H NMR Data for EMCH Hapten (4.16)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.33	**H21**	1.41	**H39**	2.18
H2'	2.58	**H21'**	1.87	**H39'**	2.18
H3	3.87	**H22**	1.60	**H40**	3.91
H4	1.35	**H22'**	1.60	**H41**	3.57
H4'	1.73	**H23**	3.53	**H42**	2.32
H5	1.38	**H24**		**CH_3-42**	0.89
H5'	2.08	**H24'**	1.69	**H43**	
H6	4.35	**H25**	2.18	**H43'**	
H7	2.94	**CH_3-25**	1.06	**H45**	
H8	4.31	**26=CH_2**	4.76	**H45'**	
H9	4.04	**26'=CH_2**	4.81	**H46**	2.17
H10	4.19	**H27**	3.53	**CH_3-46**	0.90
H11	4.59	**H28**	1.93	**H47**	3.08
H12	4.68	**H28'**		**H48**	3.55
H13	1.94	**H29**	4.20	**H49**	1.83
H13'	2.15	**H30**	4.65	**H49'**	2.16
H15	1.61	**H31**	2.01	**H50**	3.92
H15'		**CH_3-31**	0.99	**H51**	4.03
H16	1.42	**H32**	3.17	**H52**	1.97
H16'	2.17	**H33**	3.79	**H52'**	2.25
H17	4.09	**H34**	1.78	**H53**	4.71
H18	2.25	**H34'**	2.14	**H54**	7.00
H18'	2.79	**H35**	4.10	**H2'**	6.67
19=CH_2	4.91	**H36**	4.10	**H3'**	6.67
19'=CH_2	4.98	**H37**	1.88		
H20	4.36	**H37'**	2.33		

[a] The symbol ' represents the less shielded proton of a geminal pair.

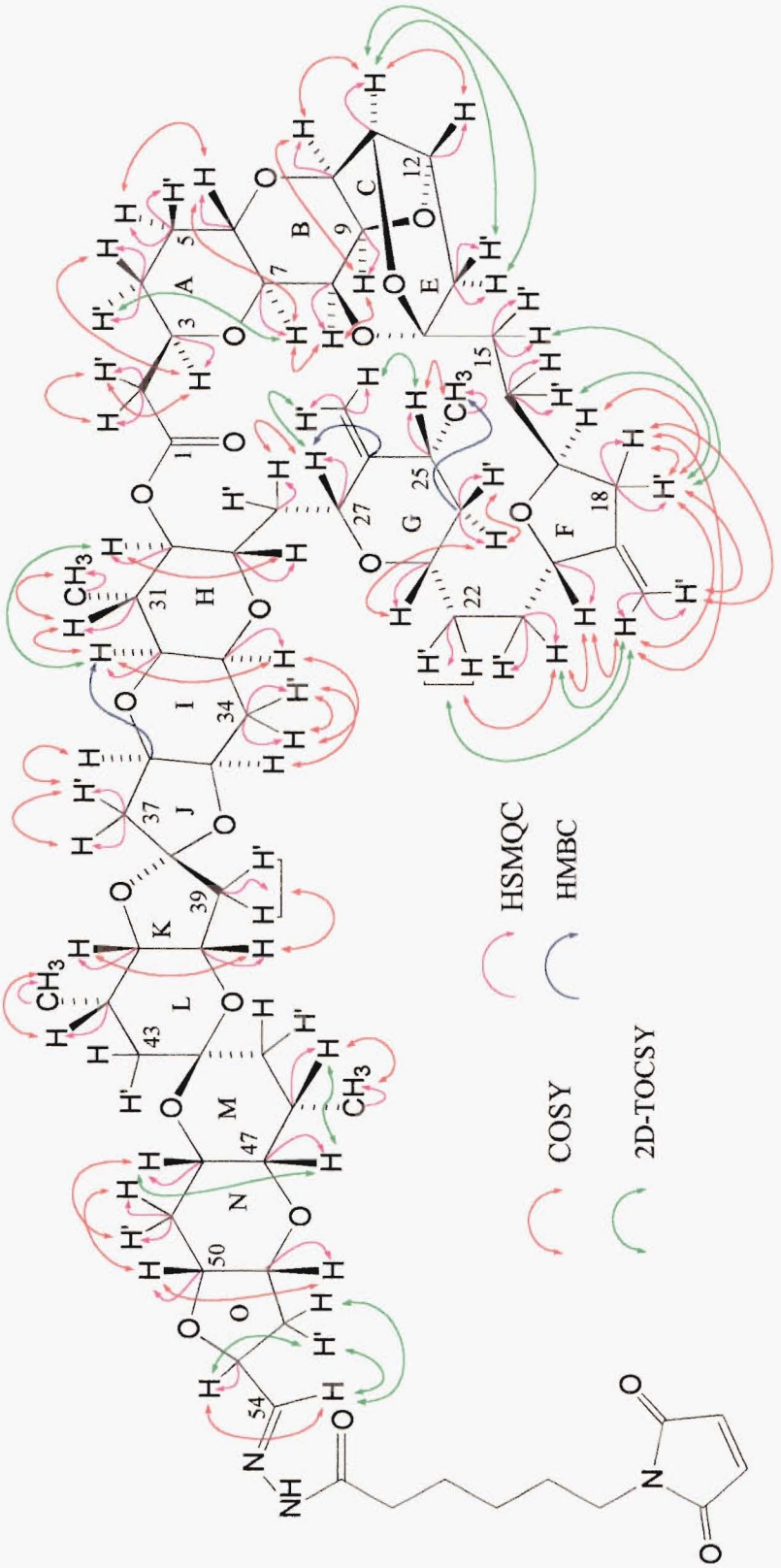
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 4.5.2 ^{13}C NMR Data for EMCH Hapten (**4.16**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1		**C21**	29.3	**C39**	42.5
C2	40.3	**C22**	31.9	**C40**	70.7
C3	73.6	**C23**	74.7	**C41**	79.3
C4	30.6	**C24**	43.4	**C**-CH_3-42	25.7
C5	30.0	**C**-CH_3-25	35.9	C-**C**H_3-42	17.1
C6	68.2	C-**C**H_3-25	17.8	**C43**	
C7	77.6	26**C**=CH_2		**C44**	
C8	74.3	26C=**C**H_2	104.1	**C45**	
C9	73.7	**C27**	73.6	**C**-CH_3-46	28.6
C10	76.5	**C28**	36.8	C-**C**H_3-46	17.4
C11	82.2	**C29**	71.1	**C47**	73.1
C12	81.1	**C30**	76.8	**C48**	63.4
C13	48.2	**C**-CH_3-31	36.6	**C49**	31.1
C14		C-**C**H_3-31	14.9	**C50**	74.6
C15	34.3	**C32**	77.5	**C51**	76.2
C16	28.0	**C33**	66.5	**C52**	38.8
C17		**C34**	29.0	**C53**	
C18	38.7	**C35**		**C54**	
19**C**=CH_2		**C36**		**C2'**	134.0
19C=**C**H_2	104.4	**C37**	43.3	**C3'**	134.0
C20	75.3	**C38**			

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from an HSMQC spectrum at 300 MHz.

Figure 4.5.2 EMCH Hapten - Important NMR Correlations



The chemical shifts and connectivities of rings A-L were identical to those of **1.25**. The doublet resonance at δ_H 7.00 was assigned as the hydrazone proton H54. The remaining protons of the halichondrin skeleton (H52-H54) were assigned from COSY and TOCSY correlations to this resonance. The H54 resonance was correlated to a resonance at δ_H 4.71 in the COSY spectrum, allowing the assignment of H53. A TOCSY correlation from the H54 resonance to δ_H 1.97 and δ_H 2.25 allowed the assignment of the H52/H52' methylene pair.

4.6 Reduction of Norhomohalichondrin B Aldehyde

4.6.1 Introduction

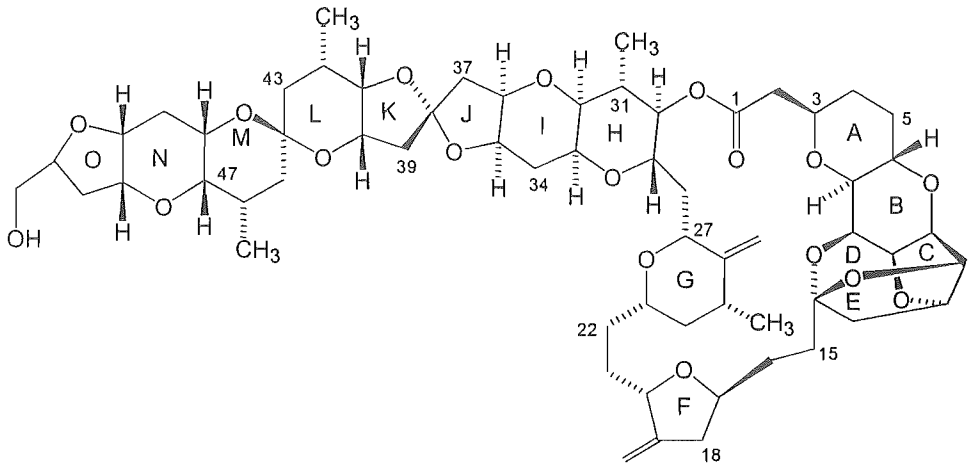
The reduction of norhomohalichondrin B aldehyde (**4.13**) to the alcohol **4.17** would facilitate the formation of a single product upon reaction of **4.17** with the phenyl or styryl linkers. This would both eliminate the purification step required when making haptens from the diol **1.25**, and improve the yield.

4.6.2 Reduction

Norhomohalichondrin B aldehyde (**4.13**, 3.9 mg) was reduced to the alcohol **4.17** upon treatment with an excess of sodium borohydride in IPA for 1 hours. The reaction was quenched with aqueous NH_4Cl, diluted with water and run through a reverse phase (C18) cartridge. The cartridge was washed with water before stripping with MeOH. The solvent was evaporated to give **4.17** (3.8 mg).

4.6.3 Structural Elucidation

The molecular formula of $C_{60}H_{84}O_{18}$ determined by HRFABMS confirmed that reduction had occurred to give **4.17**.



norhomohalichondrin B (**4.17**)

The ^{1}H NMR spectrum of **4.17** is displayed in Figure 4.6.1. The most obvious differences in the ^{1}H NMR spectrum of **4.17** compared to that of **4.13** were the absence of the H54 aldehyde resonance and the shift of the H53 resonance from an isolated position at δ_H 4.51. The region between δ_H 3.45 and δ_H 4.15 had also undergone subtle changes. A doublet of doublets resonating at δ_H 3.79 were thought to belong to the newly formed H54/H54' methylene pair.

A full array of NMR experiments were performed on **4.17** *viz* HSMQC, HMBC, 2D-TOCSY (mixing time 100 ms), COSY and APT (Figure 4.6.2) experiments. These data allowed the full assignment of the ^{1}H and ^{13}C resonances of **4.17** to be made. The ^{1}H and ^{13}C spectral data are collated in Table 4.6.1 and Table 4.6.2, and the important correlations from the 2D-NMR experiments are shown in Figure 4.6.3.

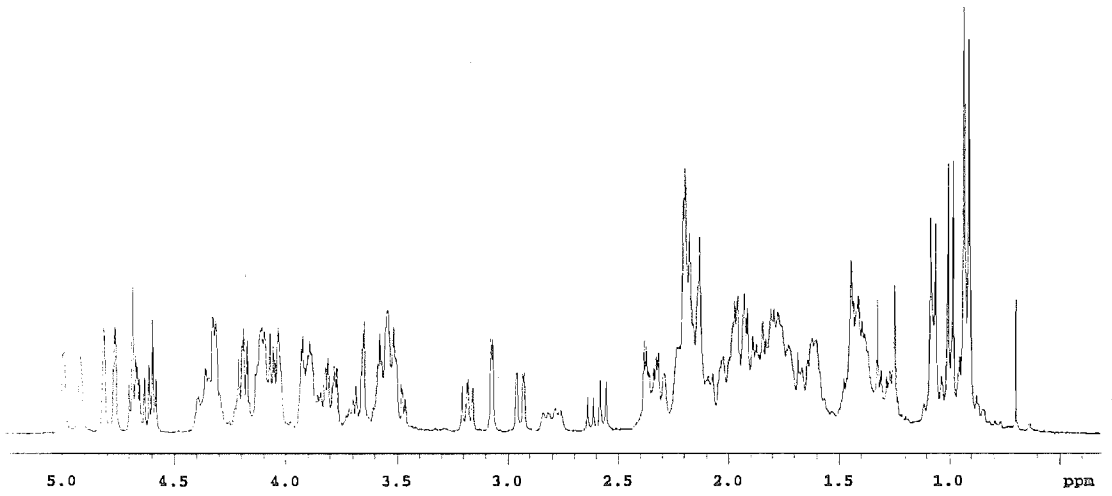


Figure 4.6.1 ^{1}H NMR Spectrum of Norhomohalichondrin B (**4.17**)

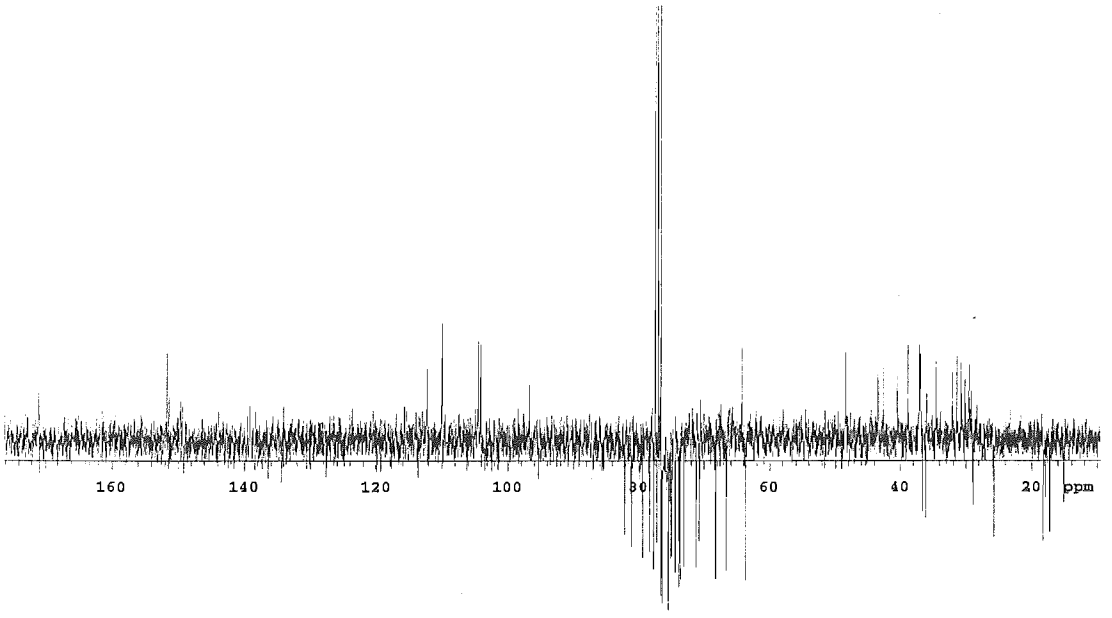


Figure 4.6.2 APT Spectrum of Norhomohalichondrin B (**4.17**)

Table 4.6.1 ^{1}H NMR Data for Norhomohalichondrin B (**4.17**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	H20	4.39	H37	1.90
H2'	2.60	H21	1.40	H37'	2.34
H3	3.88	H21'	1.89	H39	2.19
H4	1.38	H22	1.61	H39'	2.19
H4'	1.74	H22'	1.61	H40	3.93
H5	1.39	H23	3.52	H41	3.58
H5'	2.08	H24	1.03	H42	2.35
H6	4.33	H24'	1.68	CH_3-42	0.92
H7	2.94	H25	2.21	H43	1.30
H8	4.32	CH_3-25	1.07	H43'	1.43
H9	4.06	26=CH_2	4.77	H45	1.43
H10	4.18	26'=CH_2	4.81	H45'	1.43
H11	4.60	H27	3.52	H46	2.19
H12	4.69	H28	1.94	CH_3-46	0.92
H13	1.94	H28'	2.01	H47	3.07
H13'	2.14	H29	4.19	H48	3.54
H15	1.60	H30	4.66	H49	1.81
H15'	2.17	H31	2.03	H49'	2.15
H16	1.43	CH_3-31	1.00	H50	3.88
H16'	2.17	H32	3.18	H51	4.03
H17	4.10	H33	3.80	H52	1.95
H18	2.26	H34	1.79	H52'	1.95
H18'	2.80	H34'	2.15	H53	4.32
19=CH_2	4.92	H35	4.11	H54	3.49
19'=CH_2	4.99	H36	4.11	H54'	3.79

[a] The symbol ' represents the less shielded proton of a geminal pair.

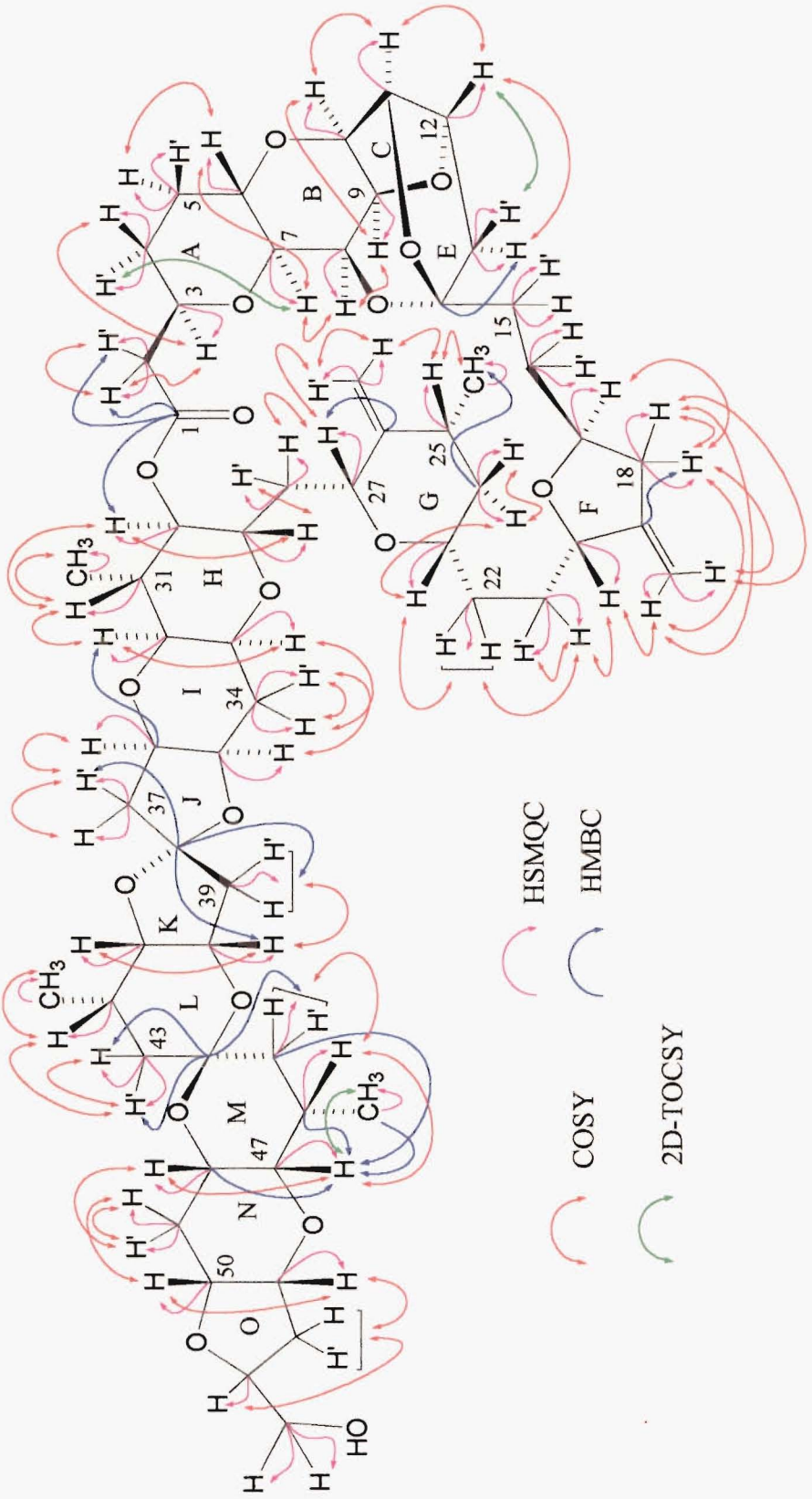
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 4.6.2 ^{13}C NMR Data for Norhomohalichondrin B (**4.17**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.1	**C20**	75.4	**C37**	43.5
C2	40.5	**C21**	29.5	**C38**	112.3
C3	73.7	**C22**	32.1	**C39**	42.6
C4	30.8	**C23**	74.8	**C40**	70.7
C5	30.1	**C24**	43.5	**C41**	79.4
C6	68.3	**$\underline{C}$-CH_3-25**	36.0	**$\underline{C}$-CH_3-42**	25.8
C7	77.7	**C-$\underline{C}H_3$-25**	18.1	**C-$\underline{C}H_3$-42**	17.8
C8	74.4	**26$\underline{C}$=CH_2**	151.7	**C43**	37.1
C9	73.9	**26C=$\underline{C}H_2$**	104.1	**C44**	96.7
C10	76.5	**C27**	73.6	**C45**	37.1
C11	82.1	**C28**	37.0	**$\underline{C}$-CH_3-46**	28.8
C12	81.1	**C29**	71.2	**C-$\underline{C}H_3$-46**	17.2
C13	48.4	**C30**	77.1	**C47**	73.1
C14	110.0	**$\underline{C}$-CH_3-31**	36.6	**C48**	63.7
C15	34.5	**C-$\underline{C}H_3$-31**	15.1	**C49**	31.5
C16	28.3	**C32**	77.6	**C50**	74.4
C17	75.3	**C33**	66.6	**C51**	77.2
C18	38.8	**C34**	29.1	**C52**	36.0
19$\underline{C}$=CH_2	151.4	**C35**	75.1	**C53**	78.3
19C=$\underline{C}H_2$	104.4	**C36**	76.3	**C54**	64.3

[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

Figure 4.6.3 Norhomohalichondrin B – Important NMR Correlations



The full assignment of the ^{1}H and ^{13}C NMR spectral data confirmed the chemical shift and connectivity of **4.17** was identical to that of **4.13** for the A-N rings. A correlation in the HSMQC spectrum between the proton resonating at δ_H 3.79 and a methylene carbon resonance at δ_C 64.1 allowed the assignment of this proton as H54'. The geminal H54 proton was assigned from the observation of a COSY correlation between the H54' resonance and a resonance at δ_H 3.49. The assignment of H54 was supported by a correlation in the HSMQC spectrum between the C54 resonance and a resonance at δ_H 3.49. A COSY correlation from both the H54 and H54' proton resonances to a resonance at δ_H 4.32 allowed the assignment of H53. The H53 resonance was correlated to a resonance at δ_H 1.95 in the COSY spectrum. The H51 resonance (δ_H 4.03) was also correlated to a resonance at δ_H 1.95 in the COSY spectrum. Both H52 and H52' were attributed to this resonance.

4.6.4 Biological Activity

The biological activity of **4.17** was evaluated in both the in-house P388 assay and the NCI's *in vitro* 60 cell line primary screening assay. The results are presented in Table 4.6.3, along with those of reference halichondrins for comparison of activity.

Table 4.6.3 *In Vitro* Cytotoxicities of Selected Halichondrins

Compound	**P388 IC_{50} (ng/mL)**	**NCI GI_{50} ($\times 10^{-10}$ M)**	**COMPARE Correlation**
halichondrin B (1.8)	0.78	1.38	1.00
homohalichondrin B (1.25)	0.22	3.16	0.95
norhomohalichondrin B (4.17)	0.64	1.23	0.99

The *in vitro* biological activity of norhomohalichondrin B (**4.17**) is comparable to that of the natural halichondrins **1.8** and **1.25**, both in the magnitude of the cytotoxicity and

in the mode of action. This is hardly suprising considering the degree of structural similarity between the three compounds.

Norhomohalichondrin B (**4.17**) has been referred to the Biological Evaluation Committee for further testing.

4.7 Norhomohalichondrin B and PMSI

4.7.1 Reaction of Norhomohalichondrin B with PMSI

Norhomohalichondrin B (2.4 mg, **4.17**) and PMSI were dissolved in $CDCl_3$ (with 0.1% CD_5N) and the reaction monitored by ^1H NMR spectroscopy. Evidence of reaction was observed after 14 hours with a reduction in the H54 resonance and changes to the methyl region of the ^1H NMR spectrum of the reaction mixture. After 42 hours the integral of the upfield styryl proton (H11', δ_H 5.95) relative to that of H32 and H18' indicated that the reaction was complete. The solvent was evaporated and the residue dissolved in CH_2Cl_2 and chromatographed on LH20 to remove excess reagent. The column was eluted with CH_2Cl_2 and thirty fractions collected. The fractions containing solely halichondrin material were identified by TLC (DIOL, 4% MeOH/CH_2Cl_2) and combined to give the product (**4.19**, 2.5 mg).

4.7.2 Structural Elucidation

The appearance of a resonance at δ_H 5.95 in the ^1H NMR spectrum of **4.19** was consistent with attachment of the PMSI linker to norhomohalichondrin B (**4.17**) (refer to Section 4.2). COSY, 2D-TOCSY (mixing time 100 ms), HSQC and HMBC experiments were performed on **4.19**. These data enabled the assignment of the majority of the ^1H and ^{13}C NMR resonances of **4.19**. The ^1H and ^{13}C NMR data are collated in Table 4.7.1 and Table 4.7.2 respectively, and the important correlations from the 2D experiments are shown in Figure 4.7.1.

Table 4.7.1 ^{1}H NMR Data for Norhomohalichondrin B PMSI Hapten (**4.19**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H22**	1.60	**H42**	2.37
H2'	2.60	**H22'**	1.60	**CH_3-42**	0.92
H3	3.88	**H23**	3.53	**H43**	1.30
H4	1.37	**H24**	1.04	**H43'**	1.43
H4'	1.73	**H24'**	1.68	**H45**	1.43
H5	1.38	**H25**	2.20	**H45'**	1.43
H5'	2.09	**CH_3-25**	1.07	**H46**	2.18
H6	4.32	**26=CH_2**	4.77	**CH_3-46**	0.92
H7	2.94	**26'=CH_2**	4.81	**H47**	3.07
H8	4.34	**H27**	3.52	**H48**	3.54
H9	4.05	**H28**	1.99	**H49**	1.80
H10	4.19	**H28'**	2.09	**H49'**	2.19
H11	4.60	**H29**	4.19	**H50**	3.91
H12	4.69	**H30**	4.66	**H51**	4.04
H13	1.93	**H31**	2.02	**H52**	1.76
H13'	2.15	**CH_3-31**	1.00	**H52'**	2.09
H15	1.61	**H32**	3.18	**H53**	4.45
H15'	2.17	**H33**	3.80	**H54**	
H16	1.42	**H34**	1.78	**H54'**	
H16'	2.18	**H34'**	2.14	**H2'**	6.84
H17	4.09	**H35**	4.11	**H3'**	6.84
H18	2.25	**H36**	4.10	**H6'**	7.24
H18'	2.80	**H37**	1.90	**H7'**	7.35
19=CH_2	4.92	**H37'**	2.34	**H9'**	7.35
19'=CH_2	4.99	**H39**	2.19	**H10'**	7.24
H20	4.37	**H39'**	2.19	**H11'**	5.95
H21	1.40	**H40**	3.91	**H12'**	7.24
H21'	1.89	**H41**	3.57		

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

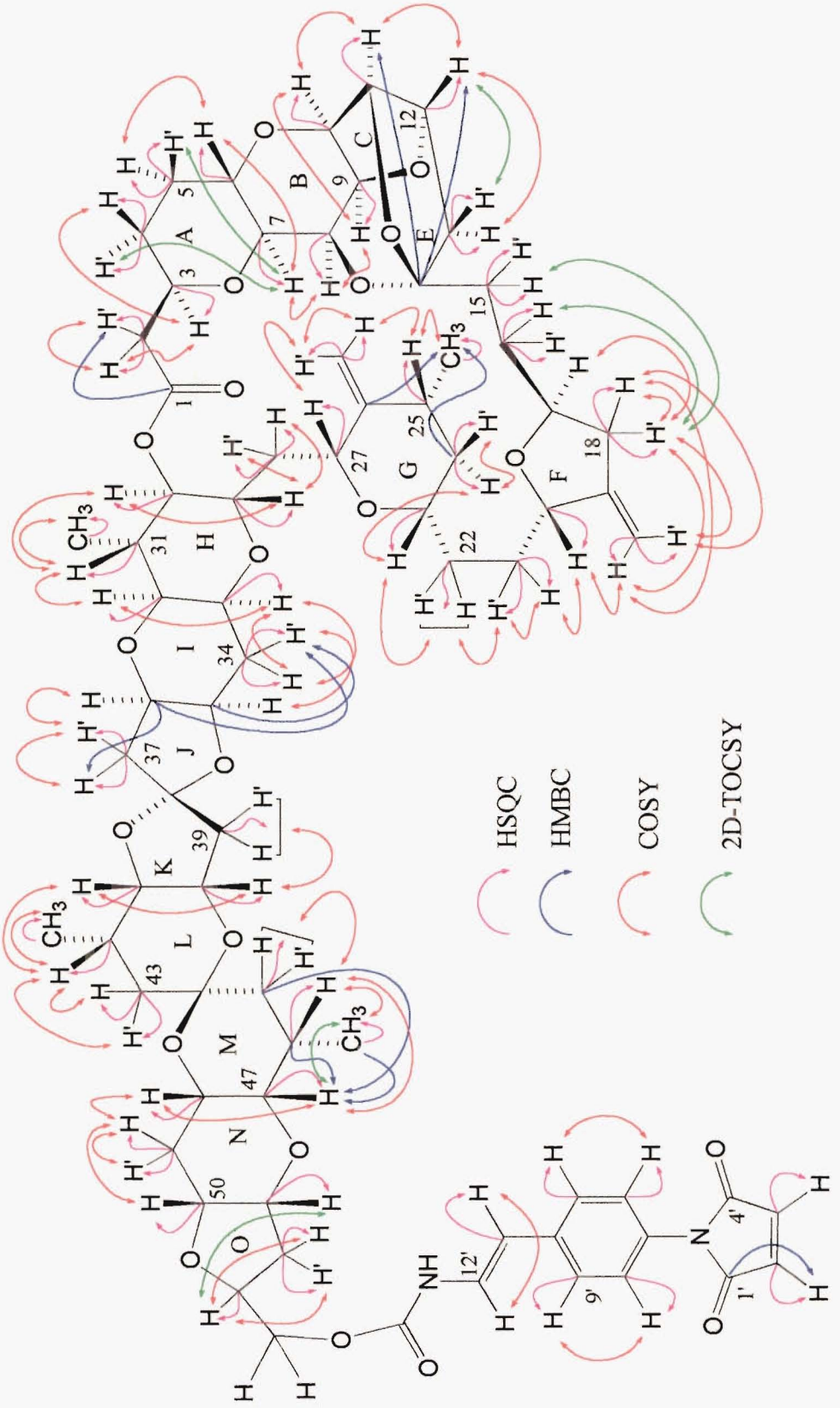
Table 4.7.2 ^{13}C NMR Data for Norhomohalichondrin B PMSI Hapten (**4.19**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.1	C-CH_3-25	35.6	C-CH_3-46	28.2
C2	40.1	C-CH_3-25	17.7	C-CH_3-46	16.9
C3	73.7	26C=CH_2	151.7	C47	73.1
C4	30.4	26C=CH_2	103.7	C48	63.5
C5	30.4	C27	73.4	C49	30.9
C6	68.0	C28	36.5	C50	74.2
C7	77.8	C29	71.2	C51	77.0
C8	74.3	C30	76.9	C52	36.5
C9	73.9	C-CH_3-31	36.5	C53	75.6
C10	76.5	C-CH_3-31	14.8	C54	
C11	81.7	C32	77.5	C1'	169.5
C12	80.6	C33	66.5	C2'	134.0
C13	48.1	C34	28.8	C3'	134.0
C14	109.9	C35	75.1[b]	C4'	169.5
C15	34.3	C36	76.2[b]	C5'	
C16	27.6	C37	43.1	C6'	126.0
C17		C38		C7'	125.8
C18	38.4	C39	42.3	C8'	
19C=CH_2		C40	70.6	C9'	126.0
19C=CH_2	104.3	C41	79.5	C10'	
C20	75.0	C-CH_3-42	25.7	C11'	109.3
C21	29.3	C-CH_3-42	16.9	C12'	147.2
C22	31.7	C43	36.8	C13'	
C23	74.7	C44			
C24	43.1	C45	36.8		

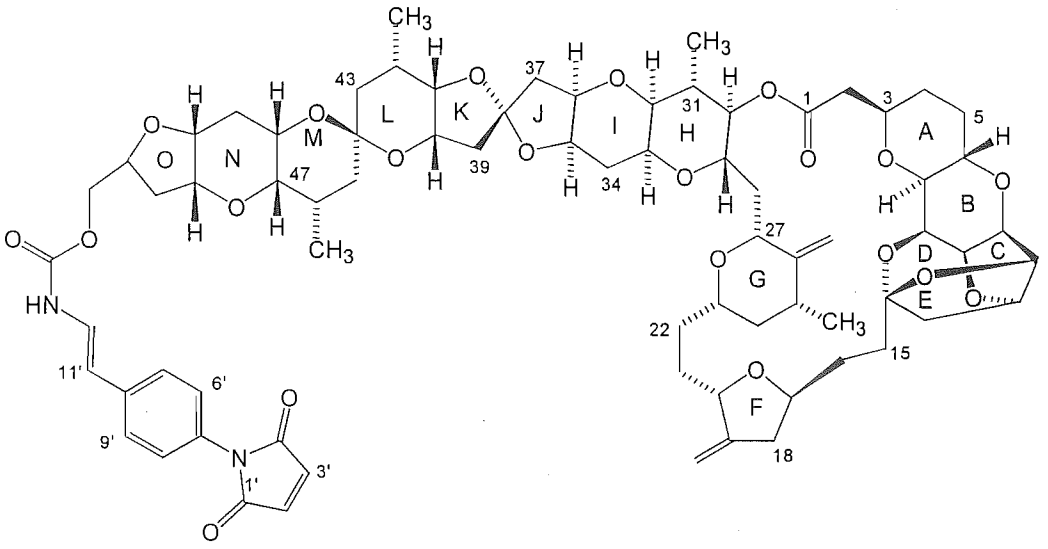
[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from an HSQC spectrum at 300 MHz.

[b] Assignment tentative – may be interchanged.

Figure 4.7.1 Norhomohalichondrin B PMSI Hapten – Important NMR Correlations



These data confirmed the connectivity and chemical shift of **4.19** were identical to that of **4.17** for the A-N rings. A methine proton at δ_H 4.45 showed a COSY correlation to a pair of methylene protons (δ_H 1.76, 2.09). A TOCSY correlation from this methine proton to the H51 proton resonance (δ_H 4.04) allowed the assignment of H53. The methylene proton resonances were assigned as H52 and H52'. The C52 resonance (δ_C 36.5), assigned from the observation of a correlation with the H52 proton in the HSQC spectrum, supports the assignment of the methylene pair as H52 and H52' rather than the oxygenated methylene protons H54 and H54'. It was not possible to assign H54 and H54' as no COSY or TOCSY correlations to the methylene pair were seen from H53.



norhomohalichondrin B PMSI hapten (**4.19**)

4.8 Summary

The formation of haptens "presenting" the macrocyclic portion of the halichondrin skeleton has been investigated. A series of haptens were constructed utilising the linkers PMSI, PMPI and EMCH attached to one or more of homohalichondrin B (**1.25**), isohomohalichondrin B (**1.28**) and norhomohalichondrin B aldehyde (**4.13**). Reduction of the aldehyde **4.13** to norhomohalichondrin B (**4.17**) provided the starting material for an improved route (in terms of yield) to a PMPI/PMSI hapten.

Chapter 5

Double Bond Alteration

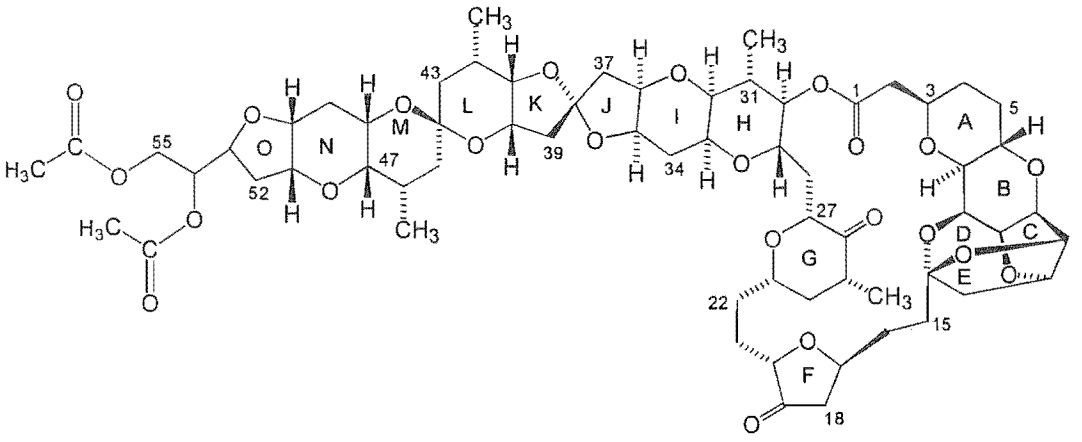
5.1 Introduction

As discussed in Section 4.1, antibodies elicited in response to an immunogenic protein conjugated distal to the terminal site of a B series halichondrin should discriminate between the B series congeners. The New Zealand sponge *Lissodendoryx* sp. is currently the most likely source of halichondrins for future preclinical trials.[62] To date only B series halichondrins have been isolated from this sponge. The development of an immunoassay with the ability to distinguish between the B series analogues would be a valuable tool for assessing the purity of drug preparations.

The possible sites for attachment of a linker distal to the terminus of the halichondrin skeleton are limited to the C1-C30 lactone and the CH_2=19 and CH_2=26 exocyclic methylenes. The lactone was not regarded as a good option, as it was preferable to maintain the integrity of the halichondrin skeleton to gain maximum antibody recognition. The exocyclic methylenes were considered the most likely site at which to achieve linker attachment.

Diketone **5.1**[95] was a readily accessible compound for reaction with the EMCH linker (Section 4.5). However attachment of a single linker at one of either C19 or C26, with retention of the remaining olefin, was preferable. The differential reactivity of the 19 and 26 exocyclic methylenes observed previously during the formation of the diketone **5.1**[95] indicated that selective derivatisation of the olefinic groups may be possible. The X-ray structure of norhalichondrin A (Figure 5.1.1)[98] and the solution conformations of

homohalichondrin B indicated by NMR spectroscopy[95] suggest that the CH_2=19 group is orientated into the lactone ring and thus is less exposed (greater steric hindrance) than the CH_2=26 group which points towards the outside of the lactone ring. The choice of a sterically demanding reagent could be expected to enhance this difference in reactivity.



homohalichondrin B diacetate diketone (**5.1**)

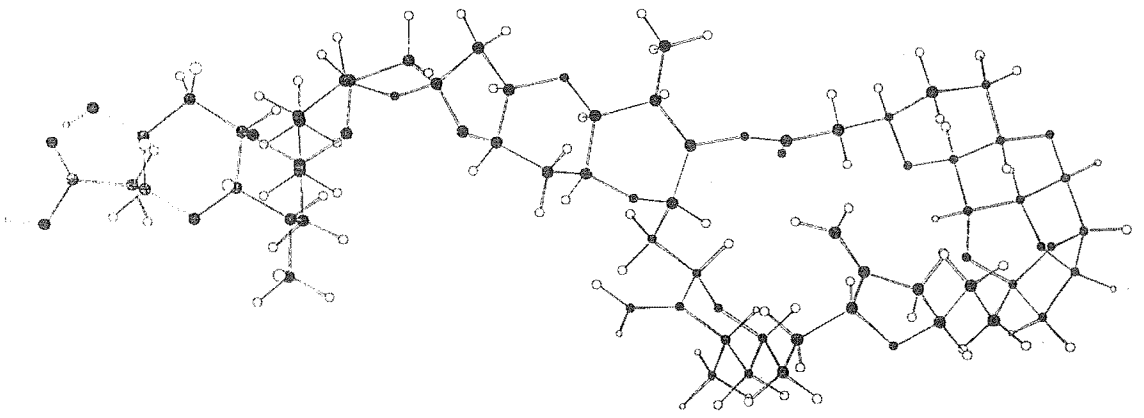


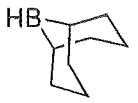
Figure 5.1.1 The X-ray Structure of Norhalichondrin A[98]

5.2 Hydroboration-oxidation

Hydroboration-oxidation of the CH_2=26 methylene would be expected to give a hydroxy derivative. Haptens could be produced from this derivative upon reaction with PMPI (**4.2**) or PMSI (**4.1**).

5.2.1 9-BBN

9-Borabicyclo[3.3.1]nonane (9-BBN, **5.2**) was chosen as the hydroborating agent because of its size and because it was readily available in a stable crystalline form.



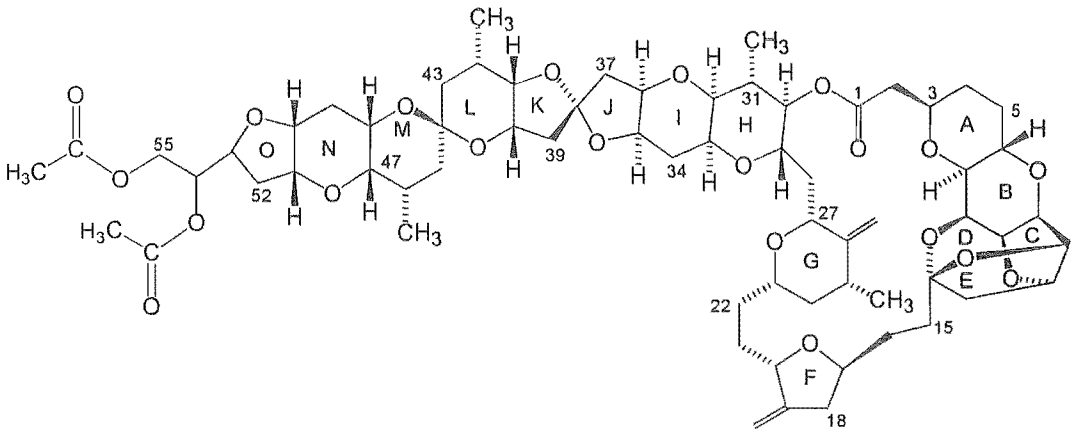
9-BBN (**5.2**)

5.2.1.1 Reaction of β-Pinene with **5.2**

The reaction conditions for hydroboration were established using β-pinene as a model compound. β-Pinene was treated with **5.2** in THF for 18 hours before the addition of a basic solution of hydrogen peroxide. The reaction was stirred for a further 2 hours before quenching with potassium carbonate. A ^{1}H NMR spectrum of the organic extract indicated the reaction was nearly complete as the exocyclic methylene resonances were barely visible.

5.2.1.2 Acetylation of **1.25**

The protection of the terminal diol of homohalichondrin B (**1.25**) was necessary before hydroboration to limit the possible sites of reaction for the PMPI (**4.2**) or PMSI (**4.1**) linkers to the newly formed alcohol. Compound **1.25** was acetylated with acetic anhydride in pyridine at room temperature for 16 hours. The reaction was quenched with H_2O before extraction with EtOAc. The ^{1}H NMR spectrum of the organic extract was identical to that of an authentic sample of homohalichondrin B diacetate (**5.3**).



homohalichondrin B diacetate (**5.3**)

5.2.1.3 Reaction of **5.3** with 9-BBN

Homohalichondrin B diacetate (**5.3**, 0.6 mg) was treated with 9-BBN in THF at *ca* 30°C for 8 hours. A basic solution of hydrogen peroxide was added and the reaction stirred for a further 2 hours. The ^{1}H NMR spectrum of the EtOAc extract indicated that the starting material **5.3** was intact.

The reaction was repeated and allowed to react for 5 days before the addition of basic hydrogen peroxide. After a further 6 hours the reaction mixture was extracted with EtOAc. The exocyclic methylene resonances were still visible in the ^{1}H NMR spectrum of the organic extract. Comparison of the integrals of these olefinic resonances with the H54 methine resonance confirmed that no reaction had occurred.

The reaction was repeated using a twenty-fold molar excess of 9-BBN to negate any influence that trace amounts of moisture might be having on the reaction. Once again starting material **5.3** was recovered. It was concluded that 9-BBN was simply too large to react with the sterically hindered exocyclic methylenes of homohalichondrin B diacetate (**5.3**).

5.2.2 Borane Dimethyl Sulfide

A smaller hydroborating agent was required. Borane dimethyl sulfide was chosen because of its size and availability. Reaction conditions were again established using β-pinene as a model compound.

5.2.2.1 Hydroboration of **5.3**

A THF solution of borane dimethyl sulfide was added to **5.3** (0.5 mg) in dry $CDCl_3$ in an NMR tube filled with argon. The reaction was monitored half hourly by ^1H NMR spectroscopy, and when no change to the exocyclic methylene resonances was observed a further aliquot of borane dimethyl sulfide (2 μL) was added. Three such additions were made with no effect on the starting material. It was thought that the scale of the reaction may be enhancing the detrimental effect of any residual water in the solvent or in the NMR tubes used. To negate this problem excess reagent was used. This did give rise to some reaction as a small change in the chemical shift and intensity of the exocyclic methylene resonances was observed, along with the disappearance of the H32 resonance. The solution was oxidised (H_2O_2/NaOH) and the product extracted with EtOAc. The complexity of the methyl region of the ^1H NMR spectrum indicated that the extract contained a mixture of products. The shift in the exocyclic methylene resonances could not be explained but if hydroboration/oxidation had occurred there should be no resonances in this region of the spectrum. There was inadequate material for further investigation.

It was decided to change the solvent used to the more conventional THF and to use reacti-vials to facilitate dry reaction conditions. Borane dimethyl sulfide was added to homohalichondrin B diacetate (**5.3**, 0.5 mg) dissolved in dry THF (100 μL) and stirred for 16 hours. Ethanol was added to destroy the borane before adding basic hydrogen peroxide. Stirring was continued for 6 hours before extracting the product with EtOAc. The absence of the CH_2=26 methylene resonances and a reduction in the CH_2=19

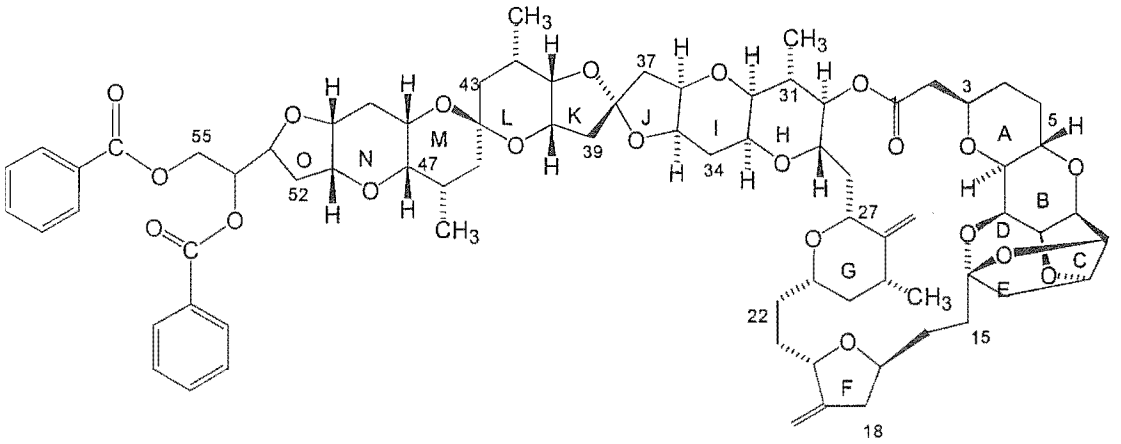
methylene resonances in the ^{1}H NMR spectrum of the extract indicated that the reaction had been successful. The incomplete derivatisation of the CH_2=19 olefin was promising as it gave an indication that it may be possible to refine the reaction conditions to enable hydroboration of the CH_2=26 methylene only. The extract was resubmitted to hydroboration/oxidation to complete oxidation of the CH_2=19 olefinic group.

It was planned to employ semipreparative reverse phase (C18) HPLC to purify the product mixture. However no peaks were observed in the HPLC chromatogram of the product mixture. The lack of UV visibility could be attributed to loss of a chromophore from the molecule, in the form of the exocyclic methylenes. TLC (DIOL, 3% MeOH/CH_2Cl_2) showed that there was a mixture of products, of similar polarity to homohalichondrin B (**1.25**). Introduction of a UV chromophore to **1.25** in the form of a phenyl-derived protecting group was necessary to enable purification of the subsequent hydroboration product mixture.

5.2.2.2 Protection of **1.25** with a UV-active Protecting Group

Several factors had to be considered when selecting a suitable protecting group. The use of acid conditions had to be avoided as previous experience had shown the halichondrins were not stable to acid treatment.[95] The halichondrins were relatively stable to dilute alkaline solutions, but less stable in the presence of more nucleophilic, Lewis bases, such as sodium methoxide.[95] Subsequent reaction of the hydroboration/oxidation product with an isocyanate linker would produce a carbamate functionality. Conditions used for the cleavage of the protecting group could not affect this coupling. The protecting group, while cleavable in mild conditions, needed to be stable to hydroboration and basic hydrogen peroxide. Careful consideration of these requirements led to the choice of benzoate ester for the protection of homohalichondrin B.

Homohalichondrin B (**1.25**, 0.3 mg) was treated with benzoic anhydride and *N*, *N*-dimethylaminopyridine (DMAP), in dry pyridine for 42 hours before extracting with EtOAc. The solvent was evaporated *in vacuo* to give a mixture of the product **5.4** and DMAP. This mixture was dissolved in water and applied to a reverse phase (C18) cartridge. The cartridge was washed with H_2O, eluted with MeOH/H_2O (1:1) to remove the DMAP and stripped with MeOH to give the product **5.4**.



homohalichondrin B dibenzoate (**5.4**)

The absence of the H55/55' methylene resonances (δ_H 3.69) and the appearance of a new resonance at δ_H 5.55 together with two full sets of phenyl-type resonances in the ^{1}H NMR spectrum of the MeOH fraction supported the assignment of homohalichondrin B dibenzoate (**5.4**). The δ_H 5.55 resonance was assigned to the H54 proton, analogous to the downfield shift observed for the H54 resonance of homohalichondrin B diacetate (**5.3**). The H54 proton resonance was irradiated in a 1D TOCSY experiment (mixing time 80 ms) to probe the chemical shifts of other protons in the same spin system. The H55/55' and H53 proton resonances were found to overlap in the region of δ_H 4.55-4.65. The upfield resonance (δ_H 1.67) was assigned to the H52/52' methylene protons.

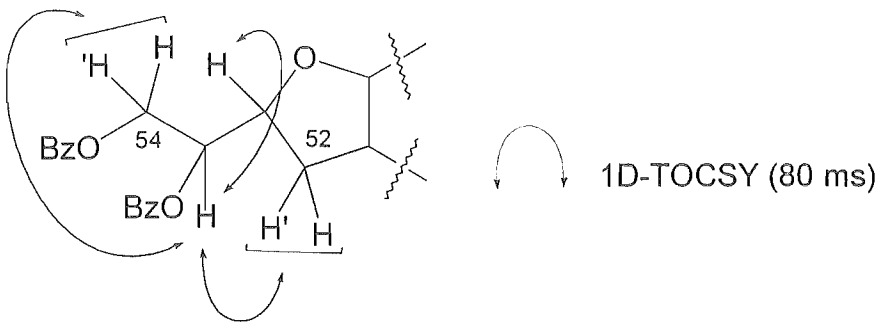


Figure 5.2.1 1D-TOCSY Correlations from H54 of **5.4**

5.2.2.3 Deprotection of **5.4**

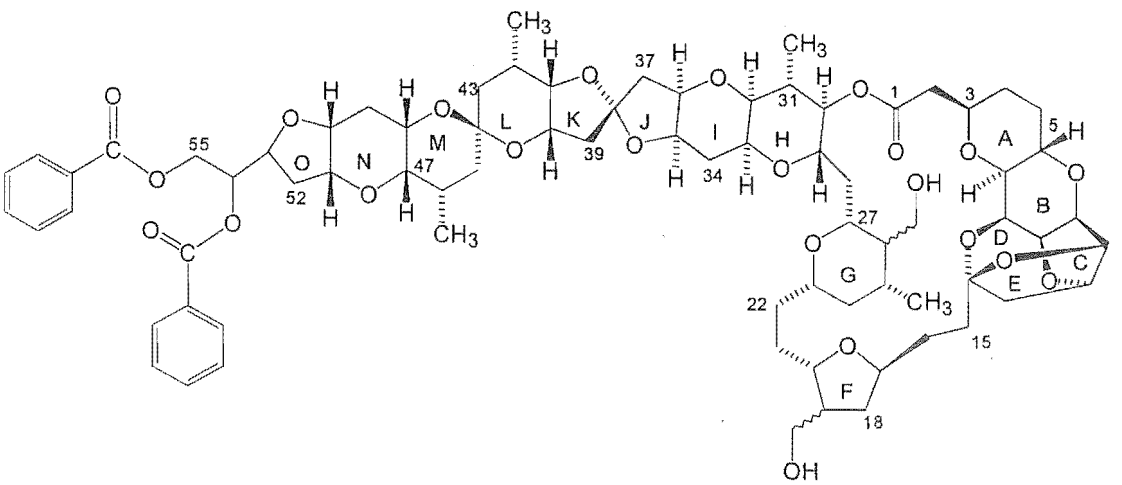
In a trial reaction to establish conditions for the cleavage of the benzoate protecting groups, the dibenzoate **5.4** was treated with 1% K_2CO_3 in MeOH for 24 hours. The removal of both benzoate protecting groups was confirmed by TLC (DIOL, 3% MeOH/CH_2Cl_2). It was realised that the use of MeOH would not be possible due to the reactivity of the maleimide linker that would be present at the deprotection step (discussed in Section 4.3.2). It was found that using acetonitrile in place of methanol gave the same result. The stability of the carbamate linker to the cleavage conditions was established using a 2,2-dimethylpropanol/PMPI derivative as a model.

5.2.2.4 Hydroboration of **5.4**

Homohalichondrin B (**1.25**, 1 mg) was protected as the dibenzoate by treatment with benzoic anhydride and DMAP in dry pyridine for 40 hours. The reaction was quenched by the addition of saturated $NaHCO_3$ and the product extracted with EtOAc. Excess DMAP was removed from the organic extract as described previously by passing it through a reverse phase (C18) cartridge. A ^1H NMR spectrum of the MeOH fraction eluted off the cartridge was comparable to the spectrum obtained previously for **5.4**.

Compound **5.4** was stirred with borane dimethyl sulfide in THF for 17 hours. The borane was quenched by the addition of a few drops of ethanol before basic hydrogen

peroxide was added. The reaction mixture was stirred for a further 6 hours before extraction of the product with EtOAc. The CH_2=19 and CH_2=19' methylene resonances were observed in the ^{1}H NMR spectrum of the product mixture. Integration of these methylene resonances relative to the H54 resonance indicated that *ca* 13% of the mono-derivatised product was present in the mixture. It appeared that both benzoate protecting groups were still intact. Reverse phase (C18) HPLC, using 60% CH_3CN/H_2O as the mobile phase, was used to separate the mixture into seven fractions, five of which were found to contain halichondrin-derived species. The absence of the characteristic exocyclic methylene resonances in the ^{1}H NMR spectrum of the most polar fraction (403 s) was consistent with the formation of a hydroboration product. The loss of one of the benzoate groups was evident from the ^{1}H NMR spectrum. A fraction of similar polarity (520 s) was found to be halichondrin in character but a lack of mass prevented a more in depth analysis. The ^{1}H NMR spectrum of the major component **5.5** (2197 s) showed that the exocyclic methylenes were no longer intact, with no resonances observed in the region of δ_H 4.75-5.05. The presence of the H54 resonance at δ_H 5.65 indicated that both benzoate protecting groups were intact. The molecular formula of $C_{75}H_{98}O_{23}$ was consistent with formation of the desired oxidation product.



hydroboration-oxidation product (**5.5**)

The complicated nature of the methyl region in the ^{1}H NMR spectrum of another non polar fraction (2353 s) indicated that it contained a mixture of dibenzoate hydroboration products. The mixture included some (50%) intact 19 methylene derivative. A further non polar fraction (2886 s) was too small to allow the deduction of anything other than its halichondrin character.

The loss of the benzoate protecting group was initially attributed to the oxidation process, which uses basic hydrogen peroxide, although benzoate protection was chosen because it is usually stable under these conditions.[99] The purity of the starting material was also questionable. A small amount of mono-protected homohalichondrin B may have been present which could have given rise to the more polar of the hydroboration products. It is impossible to be sure which of the above occurred but there are problems with both scenarios. Benzoate cleavage during oxidation would affect product yield. Alternatively, if there was a mixture of mono- and di-protected hydroxy groups in the starting material after 42 hours reaction, the ease with which the dibenzoate is formed is questionable. Benzoate protection was put to one side while other protection methods were investigated.

5.2.2.5 Protection of **1.25** with *t*-Butyldiphenylsilyl Chloride

The stability to hydroboration/oxidation conditions has been reported for *t*-butyldiphenylsilyl (TBDPS) and *t*-butyldimethylsilyl protected compounds.[100,101] A common method for the removal of this class of protecting group is treatment with HF/pyridine in THF.

Homohalichondrin B (1.2 mg) was stirred with TBDPSCl and pyridine for 42 hours at room temperature. The reaction mixture was diluted with hexanes/toluene (1:1) and loaded onto a DIOL cartridge. The cartridge was eluted with a gradient elution scheme, from hexanes/toluene (1:1) through to CH_2Cl_2. Halichondrin-type resonances were observed in the ^{1}H NMR spectra of the toluene and toluene/CH_2Cl_2 (1:1) fractions. TLC

(DIOL, 3% MeOH/CH_2Cl_2) of these combined fractions produced two spots that coloured brown with PMA spray, characteristic of halichondrin compounds. Semipreparative reverse phase (C18) HPLC using a mobile phase of 60% CH_3CN/H_2O was used to separate the mixture into three fractions. No halichondrin-like material was found in any of these fractions. The HPLC column was stripped with MeOH. A ^1H NMR spectrum of the column strip contained halichondrin-derived components. The complicated nature of the methyl region indicated that more than one compound was present. The characteristic H55/H55' methylene resonance was no longer visible, consistent with reaction of the diol group.

The difficulties encountered with the ease and reliability of protection, and subsequently deprotection, of homohalichondrin B lead to the investigation of alternative methods for exocyclic methylene derivatisation.

5.3 Osmium tetroxide

The reaction of homohalichondrin B diacetate (**5.3**) with osmium tetroxide followed by cleavage with sodium periodate to produce the 19,26-diketone **5.1** was established by Hart.[95] Evidence of differential reactivity between the CH_2=19 and CH_2=26 olefinic groups was observed. It was thought that the reaction of **5.3** with just one molar equivalent of osmium tetroxide might result in selective oxidation of the CH_2=26 olefin.

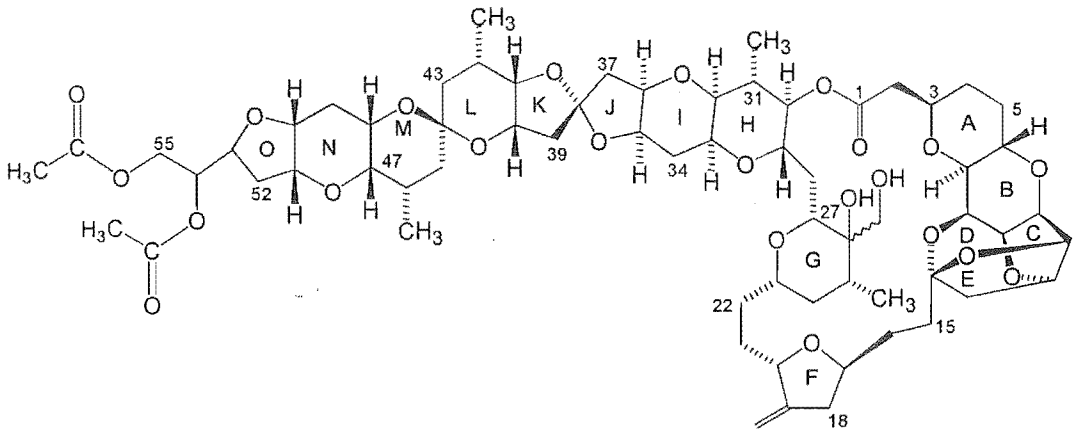
5.3.1 Reaction of 5.3 with OsO_4

Homohalichondrin B diacetate (5.3, 3.4 mg) was stirred with an ethereal solution of osmium tetroxide in pyridine for 4.5 hours. Sodium metabisulfite in water/pyridine was added to the reaction mixture and stirring continued for a further 30 minutes. The product was extracted with CH_2Cl_2 and dried over $MgSO_4$. The observation of the H54 resonance (δ_H 5.14) in the ^1H NMR spectrum of the product mixture indicated the acetate protecting groups were still intact. The CH_2=26 resonances were completely

absent, and the CH_2=19 olefinic resonances were reduced to *ca* 60% of their original area relative to **5.3**, comparing their integral to that of the H54 resonance. The appropriate resonances for the osmate esters[95] (δ_H 4.56, 4.35, 4.61, 4.74) were not observed which suggested that the diol had been formed. TLC (DIOL, 3% MeOH/CH_2Cl_2) showed the mixture contained two products, of similar polarity to homohalichondrin B (**1.25**). The product mixture was purified on DIOL, eluting with hexanes/toluene (1:1), toluene, toluene/CH_2Cl_2 (1:1) and CH_2Cl_2. Fractions identified by TLC as containing solely the diol **5.6** were combined (1.2 mg). The remaining halichondrin-containing fractions were combined and re-osmylated to produce the tetraol **5.7** (1.5 mg).

5.3.2 Characterisation of 5.6

The molecular formula for **5.6** of $C_{65}H_{92}O_{23}$ determined by HRFABMS was consistent with diol formation.



homohalichondrin B diacetate 26-diol (**5.6**)

There were several notable differences between the ^{1}H NMR spectrum of **5.6** (Figure 5.3.1) and that of **5.3**. The most significant difference was the absence of the CH_2=26 olefinic resonances. A change in chemical shift was seen for the H2' methylene resonance (upfield δ_H 0.1) and the 25-CH_3 resonance (upfield δ_H 0.06). New resonances were observed at δ_H 3.38 and *ca* δ_H 4.2.

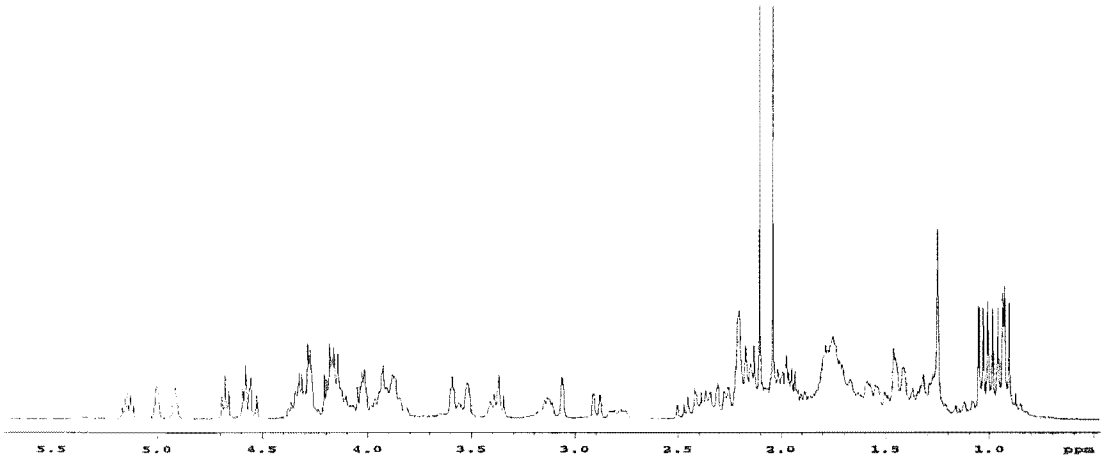


Figure 5.3.1 ^{1}H NMR Spectrum of Homohalichondrin B Diacetate 26-Diol (**5.6**)

A full array of NMR experiments were performed on **5.6** *viz* COSY, 2D-TOCSY (mixing time 100 ms), HSMQC and HMBC experiments. These data enabled the assignment of the majority of the ^{1}H NMR and ^{13}C NMR resonances of **5.6** to be achieved. The ^{1}H and ^{13}C NMR data are collated in Table 5.3.1 and Table 5.3.2, and the important correlations from the 2D experiments are shown in Figure 5.3.2.

Table 5.3.1 ^{1}H NMR Data for Homohalichondrin Diacetate 26-Diol (**5.6**)

Proton[a]	δ ppm[b]	**Proton**[a]	δ ppm[b]	**Proton**[a]	δ ppm[b]
H2	2.28	**H21**	1.43	**H39**	2.20
H2'	2.45	**H21'**		**H39'**	2.20
H3	3.84	**H22**		**H40**	3.92
H4	1.30	**H22'**		**H41**	3.60
H4'	1.66	**H23**	3.56	**H42**	2.35
H5	1.35	**H24**	1.11	**CH_3-42**	0.95
H5'	2.09	**H24'**	1.56	**H43**	1.30
H6	4.24	**H25**	1.77	**H43'**	1.45
H7	2.89	**CH_3-25**	1.00	**H45**	1.45
H8	4.29	**26-CH_2**	3.38	**H45'**	1.45
H9	4.03	**26'-CH_2**	3.88	**H46**	2.15
H10	4.18	**H27**	3.15	**CH_3-46**	0.92
H11	4.58	**H28**	1.84	**H47**	3.06
H12	4.68	**H28'**	2.03	**H48**	3.52
H13	1.96	**H29**	4.17	**H49**	1.74
H13'	2.13	**H30**	4.56	**H49'**	2.15
H15	1.60	**H31**	1.96	**H50**	3.88
H15'	2.20	**CH_3-31**	1.04	**H51**	4.01
H16	1.39	**H32**	3.37	**H52**	1.74
H16'		**H33**	3.94	**H52'**	1.98
H17	4.10	**H34**		**H53**	4.35
H18	2.24	**H34'**		**H54**	5.13
H18'	2.78	**H35**		**H55**	4.18
19=CH_2	4.92	**H36**	4.14	**H55'**	4.28
19'=CH_2	5.00	**H37**	1.90	**54OCOCH_3**	2.10
H20	4.31	**H37'**	2.37	**55OCOCH_3**	2.04

[a] The symbol ' represents the less shielded proton of a geminal pair.

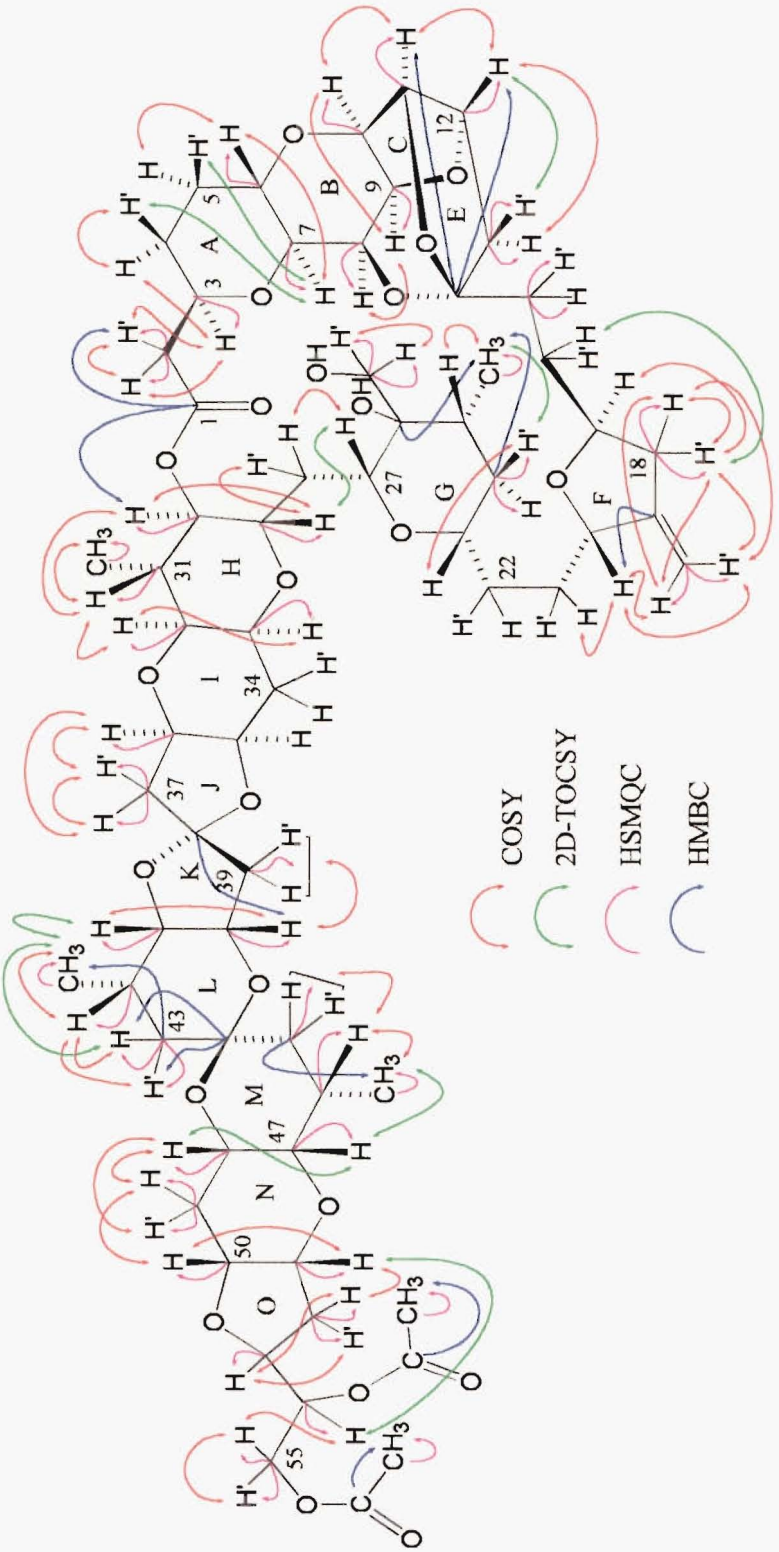
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 5.3.2 ^{13}C NMR Data for Homohalichondrin Diacetate 26-Diol (**5.6**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.2	C22		C41	79.4
C2	40.1	C23	75.3	C-CH_3-42	25.4
C3	74.6	C24	38.6	C-CH_3-42	17.2
C4		C-CH_3-25	40.8	C43	37.1
C5	29.8	C-CH_3-25	14.7	C44	96.3
C6	68.0	26C-CH_2	70.9	C45	36.7
C7	77.8	26C-CH_2	58.3	C-CH_3-46	28.5
C8	74.0	C27	79.3	C-CH_3-46	16.9
C9	73.7	C28		C47	72.7
C10	76.5	C29	69.0	C48	63.6
C11	82.2	C30	74.3	C49	31.0
C12	80.9	C-CH_3-31	37.6	C50	74.3
C13	48.3	C-CH_3-31	15.4	C51	76.2
C14	109.5	C32	75.9	C52	36.4
C15	34.5	C33	68.0	C53	76.2
C16		C34		C54	72.1
C17	75.6	C35	75.9	C55	63.7
C18	38.9	C36	75.9	54O$COCH_3$	170.6
19C=CH_2	151.9	C37	43.6	54OCOCH_3	20.7
19C=CH_2	103.8	C38	112.3	55O$COCH_3$	170.6
C20	74.0	C39	42.3	55OCOCH_3	20.7
C21		C40	70.6		

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from HSMQC and HMBC spectra at 300 MHz.

Figure 5.3.2 Homohalichondrin B Diacetate 26-Diol – Important NMR Correlations



Significant changes in chemical shift were observed for the H20 to H33 proton resonances. The connectivity and chemical shifts of the remaining protons were readily established using the information obtained from the COSY and 2D-TOCSY experiments. Correlations in the COSY spectrum from the downfield H30 resonance (δ_H 4.56) to protons resonating at δ_H 4.17 and δ_H 1.96 allowed the assignment of H29 and H31 respectively. A COSY correlation between the H31 resonance and δ_H 1.04 allowed the assignment of CH_3-31. This allowed the remaining methyl resonance (δ_H 1.00) to be assigned to CH_3-25. The H31 resonance was correlated to δ_H 3.37 in the COSY spectrum, allowing the assignment of H32. A TOCSY correlation between the H30 resonance and δ_H 3.15 allowed the assignment of H27. H28 (δ_H 1.84) and H28' (δ_H 2.03) were assigned from the observation of COSY correlations between these methylene resonances and the H27 resonance (δ_H 3.15). A COSY correlation between the CH_3-25 methyl resonance and δ_H 1.77 allowed the assignment of H25. TOCSY correlations were observed between the CH_3-25 resonance and δ_H 1.77 (H25), δ_H 1.56 and δ_H 3.56 allowing the assignment of H24' and H23 respectively. A COSY correlation to the H24' resonance from δ_H 1.11 allowed the assignment of H24. A correlation in the HSMQC between a pair of methylene proton resonances (δ_H 3.38 and δ_H 3.88) and a carbon resonating at δ_C 58.3 allowed the assignment of CH_2-26 and CH_2-26'.

5.3.3 Further Osmylation of 5.3

The osmylation reaction was repeated on a larger scale, using a freshly prepared batch of **5.3**, to check the reproducibilty of the result. The reaction was to provide material for complete characterisation of the 26-keto derivative, for methylenation attempts, and for future hapten production should this be required. Homohalichondrin B (**1.25**, 14.4 mg) was stirred with acetic anhydride in dry pyridine for 16 hours at room temperature. The reaction was quenched with water and extracted with EtOAc to give the diacetate **5.3** (15.4 mg).

The protected halichondrin **5.3** was stirred with osmium tetroxide in pyridine for 4.5 hours. Sodium metabisulfite in water/pyridine was added and the reaction stirred for a further 20 minutes. A ^{1}H NMR spectrum after work-up showed that *ca* 30% of the product mixture contained intact CH_2=19 olefin. This result is possibly attributable to the difficulties involved in adding small volumes of the OsO_4 solution.

The product mixture was purified by normal phase (DIOL) chromatography with elution by toluene through to CH_2Cl_2. The fractions were analysed using ^{1}H NMR spectroscopy as the lack of a UV chromophore precluded the use of analytical HPLC. The ^{1}H NMR spectra of several fractions contained two pairs of exocyclic methylene resonances. Only a single pair of resonances for the CH_2=19 methylene protons was observed for the product mixture before DIOL chromatography. This would seem to indicate that some change had occurred during the chromatography.

Fractions 14, 15 and 16, eluted with CH_2Cl_2, were combined on the basis of their identical ^{1}H NMR spectra to give **5.7** (3.4 mg).

5.3.4 Characterisation of 5.7

The ^{1}H NMR spectrum of **5.7** is displayed in Figure 5.3.3. The most obvious difference between this spectrum and that of the starting material **5.3** is the absence of the 19 and 26 methylene resonances. The H18' resonance is no longer clearly visible, and there are additional resonances in the region of δ_H 3.3-3.5.

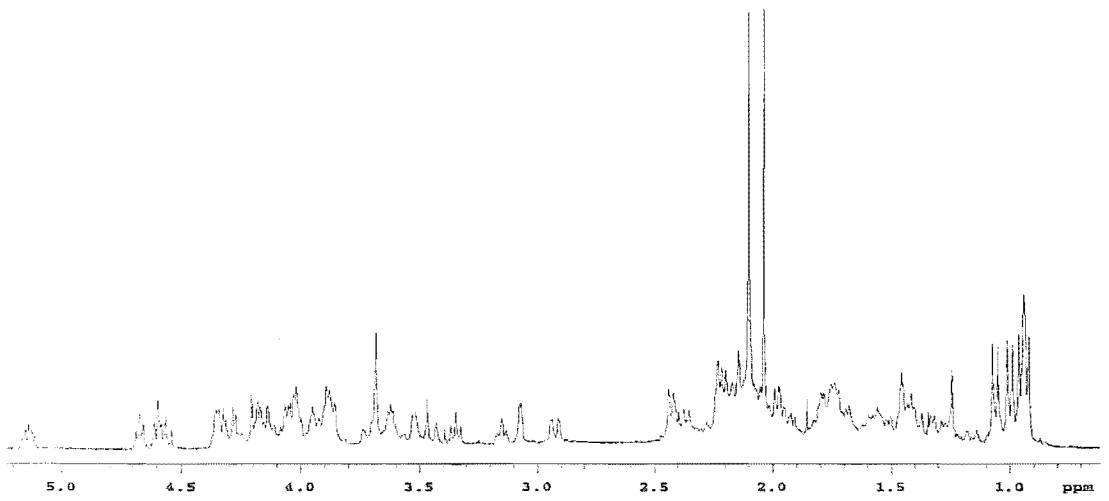


Figure 5.3.3 ^{1}H NMR Spectrum of Homohalichondrin B Diacetate 19,26-Tetraol (**5.7**)

An array of 2D NMR experiments were performed on **5.7** *viz* COSY, TOCSY (mixing time 80 ms), HSMQC and HMBC experiments. The ^{1}H and ^{13}C NMR data extracted from these experiments are listed in Table 5.3.3 and Table 5.3.4 respectively. The important correlations from the 2D experiments are shown in Figure 5.3.4.

Table 5.3.3 ^{1}H NMR Data for Homohalichondrin B Diacetate 19,26-Tetraol (**5.7**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.42	**H21**	1.68	**H39**	2.21
H2'	2.42	**H21'**	1.81	**H39'**	2.21
H3	3.91	**H22**	1.56	**H40**	3.95
H4	1.37	**H22'**		**H41**	3.61
H4'	1.73	**H23**	3.61	**H42**	2.36
H5	1.39	**H24**	1.15	**CH_3-42**	0.95
H5'	2.11	**H24'**	1.57	**H43**	1.30
H6	4.25	**H25**	1.77	**H43'**	1.43
H7	2.93	**CH_3-25**	1.00	**H45**	1.44
H8	4.35	**26-CH_2**	3.45	**H45'**	1.44
H9	4.06	**26'-CH_2**	3.87	**H46**	2.15
H10	4.18	**H27**	3.15	**CH_3-46**	0.93
H11	4.60	**H28**	1.75	**H47**	3.07
H12	4.67	**H28'**	2.15	**H48**	3.52
H13	1.96	**H29**	4.01	**H49**	1.75
H13'	2.11	**H30**	4.57	**H49'**	2.15
H15	1.56	**H31**	2.06	**H50**	3.87
H15'	2.26	**CH_3-31**	1.06	**H51**	4.02
H16	1.45	**H32**	3.36	**H52**	1.75
H16'		**H33**	3.91	**H52'**	1.98
H17	4.12	**H34**	2.09	**H53**	4.35
H18	1.71	**H34'**	2.09	**H54**	5.14
H18'	2.21	**H35**	4.14	**H55**	4.18
19-CH_2	3.70	**H36**	4.06	**H55'**	4.27
19'-CH_2	3.70	**H37**	1.93	**54OCOCH_3**	2.10
H20	3.72	**H37'**	2.38	**55OCOCH_3**	2.04

[a] The symbol ' represents the less shielded proton of a geminal pair.

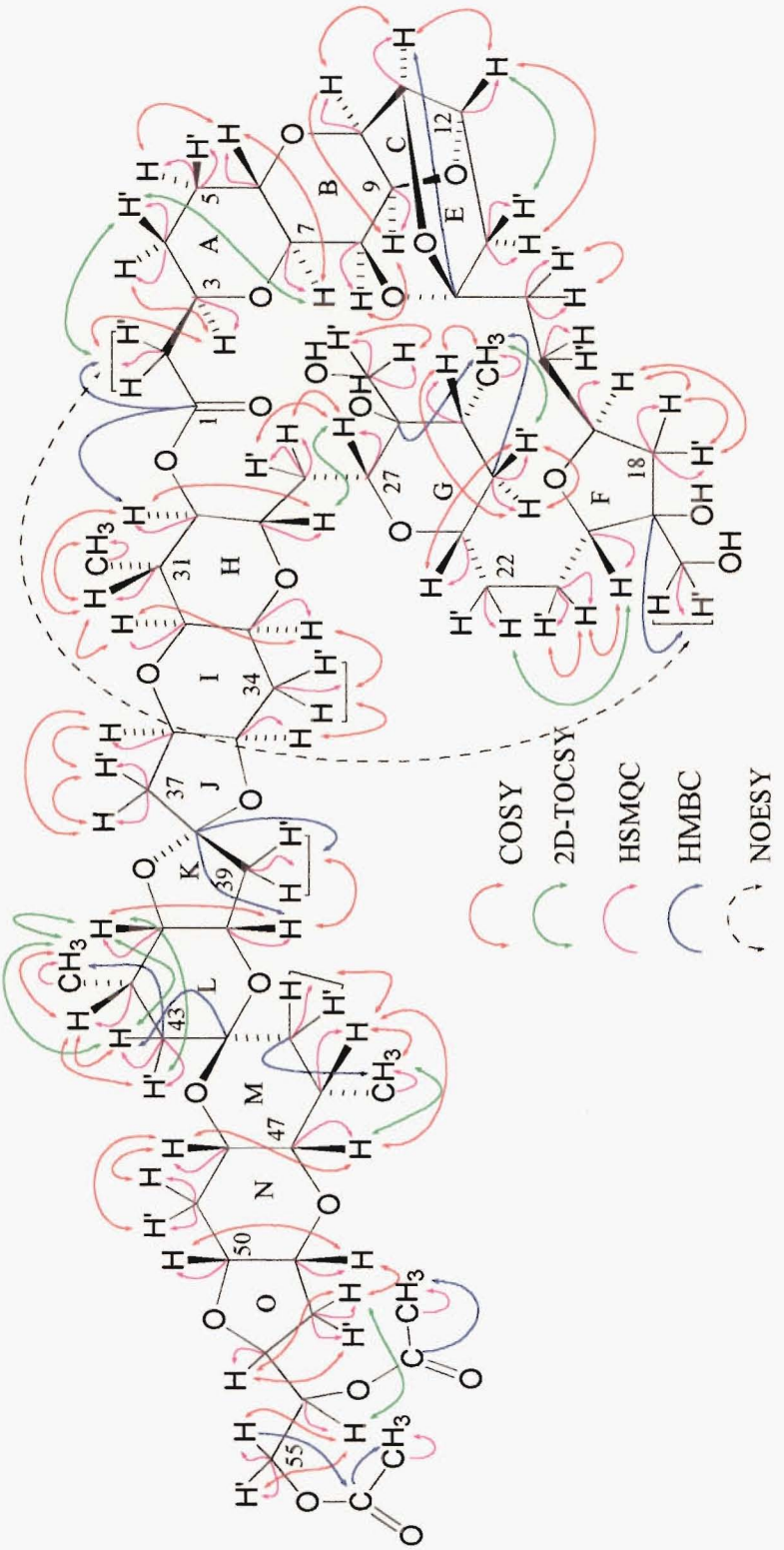
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 5.3.4 ^{13}C NMR Data for Homohalichondrin B Diacetate 19,26-Tetraol (**5.7**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	168.7	C22	30.6	C41	79.5
C2	40.8	C23	75.3	C-CH_3-42	25.4
C3	74.8	C24	38.8	C-CH_3-42	17.4
C4	30.6	C-CH_3-25	40.8	C43	36.9
C5	30.1	C-CH_3-25	14.7	C44	96.4
C6	68.2	26C-CH_2	71.0	C45	36.9
C7	78.1	26C-CH_2	59.1	C-CH_3-46	28.7
C8	74.2	C27	79.8	C-CH_3-46	16.9
C9	73.7	C28	30.6	C47	72.9
C10	76.4	C29	79.8	C48	63.5
C11	82.2	C30	75.1	C49	30.9
C12	80.6	C-CH_3-31	36.9	C50	74.2
C13	48.4	C-CH_3-31	15.5	C51	76.2
C14	108.9	C32	75.9	C52	36.3
C15	35.3	C33	67.1	C53	76.2
C16	28.7	C34	27.6	C54	72.3
C17	74.5	C35	75.9	C55	63.8
C18	44.9	C36	75.6	54O$COCH_3$	170.4
19C-CH_2	81.2	C37	44.0	54OCOCH_3	20.7
19C-CH_2	67.4	C38	112.5	55O$COCH_3$	170.4
C20	77.5	C39	42.9	55OCOCH_3	20.4
C21	23.2	C40	71.0		

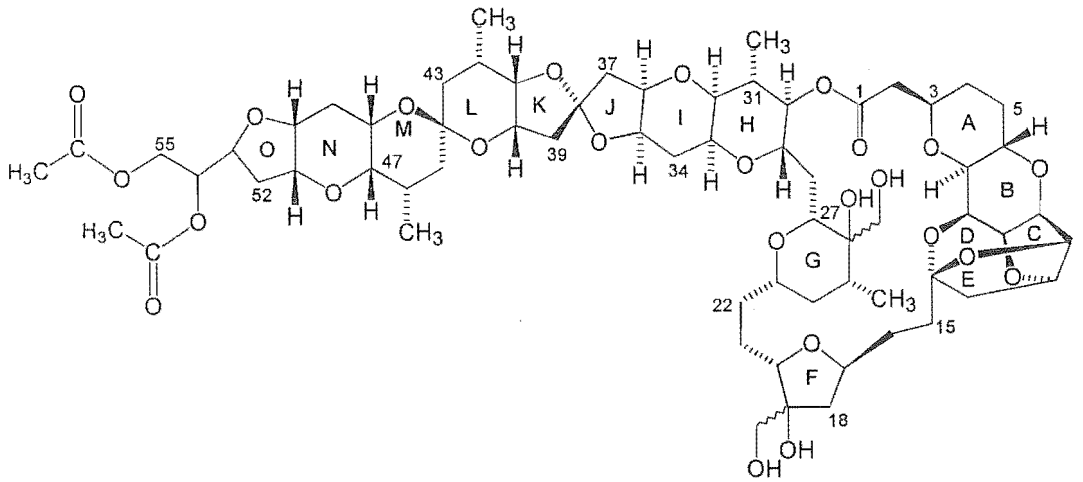
[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from HSMQC and HMBC spectra at 300 MHz.

Figure 5.3.4 Homohalichondrin B Diacetate 19,26-Tetraol – Important NMR Correlations



The NMR data were consistent with those of the diol **5.6** in the region of the A-E and K-N rings. Some similarities in chemical shift were observed in the region of the 26-diol, but differences ($\geq \delta_H$ 0.05) were seen for the H28, H28', H29, H31 and H33 resonances. This may reflect the proximity of these protons to the 19-diol. The CH_2-19 and CH_2-19' proton resonances (δ_H 3.90) showed HMBC correlations to C19 (δ_C 81.2), C18 (δ_C 44.9) and C20 (δ_C 77.5) resonances. A COSY correlation between the H20 resonance (δ_H 3.72) and a resonance at δ_H 1.68 allowed the assignment of H21. The H21' resonance (δ_H 1.81) was assigned from the observation of a COSY correlation between this proton resonance and the geminal H21 resonance. TOCSY correlations from the H20 resonance were seen to the resonances of H21, H21', H22 and H23.

The molecular formula of $C_{65}H_{94}O_{25}$, determined by HRFABMS, is consistent with the formation of **5.7**.



homohalichondrin B diacetate 19,26-tetraol (**5.7**)

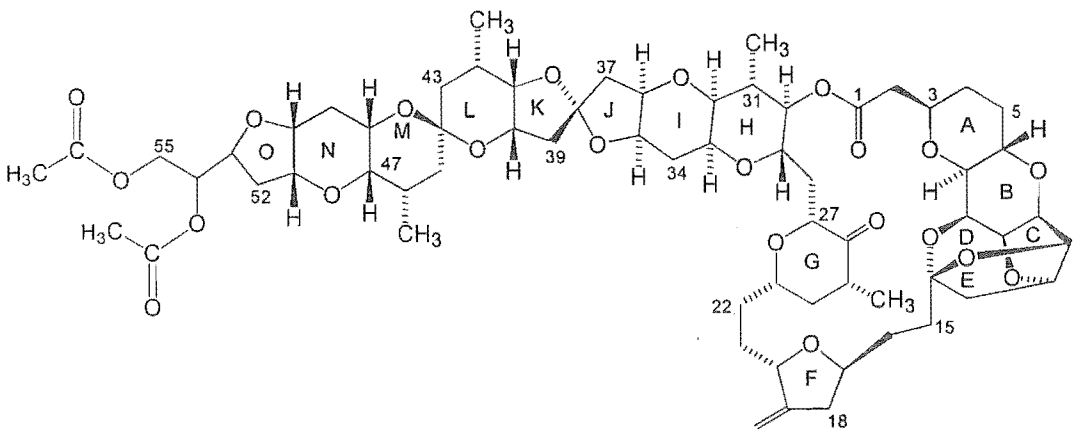
5.3.5 Periodate cleavage of osmylation products

A small amount (0.3 mg) of **5.6** was retained for deprotection (Section 5.3.10). The remaining 0.9 mg was treated with 1.2 equivalents of aqueous $NaIO_4$ for 15 hours. Fractions containing intact CH_2=19 olefinic groups, obtained from the second osmylation reaction (Section 5.3.3), were combined (2.7 mg) and treated likewise with

$NaIO_4$ for 16 hours. The products were separately extracted with EtOAc. The complex nature of the methyl region of the ^1H NMR spectra of the EtOAc extracts indicated the presence of a mixture of products. Analytical reverse phase (C18) HPLC, using a mobile phase of 60% CH_3CN/H_2O, showed that three halichondrin-type components were present in the product mixtures. Two components were of similar polarity (419 s and 595 s), and the third component was significantly less polar (1340 s). The **5.6** derived product mixture contained predominantly the more polar component, with trace amounts of the other two components. Semipreparative reverse phase (C18) HPLC, using 80% CH_3CN/H_2O as the mobile phase, was used to separate the product mixtures into three fractions **5.8** (2.0 mg), **5.9** (0.8 mg) and **5.10** (~ 0.1 mg).

5.3.6 Characterisation of 5.8

The ^1H NMR spectrum of **5.8** (Figure 5.3.5) showed several changes relative to the spectrum of the diol **5.6**. The CH_3-25 doublet was apparent at δ_H 1.11, in a less shielded position relative to **5.6** (δ_H 1.00), as expected for ketone formation. Resonances were no longer observed in the region of δ_H 3.35-3.50 as the CH_2-26 resonance was absent and the H32 resonance had shifted to a more shielded position (δ_H 3.12). A new isolated resonance was observed at δ_H 4.45.



homohalichondrin B diacetate 26-ketone (**5.8**)

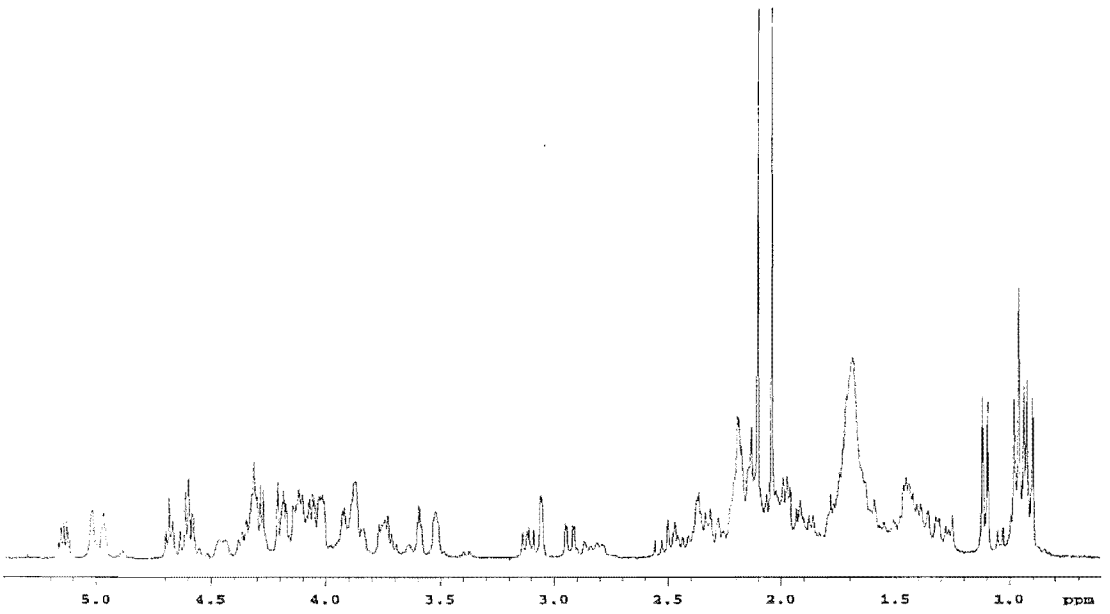


Figure 5.3.5 ^{1}H NMR Spectrum of Homohalichondrin Diacetate 26-Ketone (**5.8**)

A range of NMR experiments (COSY, 2D-TOCSY (mixing time 100 ms), HSMQC, HMBC, ^{13}C and NOE) were performed on the ketone **5.8**. The ^{1}H and ^{13}C NMR data obtained from these experiments are listed in Table 5.3.5 and Table 5.3.6. The important NMR correlations from these experiments are shown in Figure 5.3.6.

Table 5.3.5 ^{1}H NMR Data for Homohalichondrin B Diacetate 26-Ketone (**5.8**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.33	**H21**	1.48	**H40**	3.90
H2'	2.49	**H21'**	1.85	**H41**	3.59
H3	3.87	**H22**	1.70	**H42**	2.35
H4	1.35	**H22'**	1.70	**CH_3-42**	0.95
H4'	1.67	**H23**	3.86	**H43**	1.30
H5	1.35	**H24**	1.60	**H43'**	1.42
H5'	2.06	**H24'**	2.02	**H45**	1.42
H6	4.29	**H25**	2.43	**H45'**	1.42
H7	2.93	**CH_3-25**	1.11	**H46**	2.13
H8	4.33	**H27**	3.74	**CH_3-46**	0.91
H9	4.08	**H28**		**H47**	3.06
H10	4.20	**H28'**		**H48**	3.52
H11	4.60	**H29**	4.05	**H49**	1.75
H12	4.68	**H30**	4.62	**H49'**	2.16
H13	1.94	**H31**	1.95	**H50**	3.84
H13'	2.15	**CH_3-31**	0.97	**H51**	4.05
H15	1.61	**H32**	3.12	**H52**	1.75
H15'	2.20	**H33**	3.73	**H52'**	1.99
H16	1.40	**H34**	1.68	**H53**	4.35
H16'	2.13	**H34'**	2.13	**H54**	5.14
H17	4.08	**H35**		**H55**	4.17
H18	2.24	**H36**	4.12	**H55'**	4.28
H18'	2.83	**H37**	1.88	**54OCOCH$_3$**	2.10
19=CH_2	4.97	**H37'**	2.34	**55OCOCH$_3$**	2.04
19'=CH_2	5.02	**H39**	2.17		
H20	4.45	**H39'**	2.17		

[a] The symbol ' represents the less shielded proton of a geminal pair.

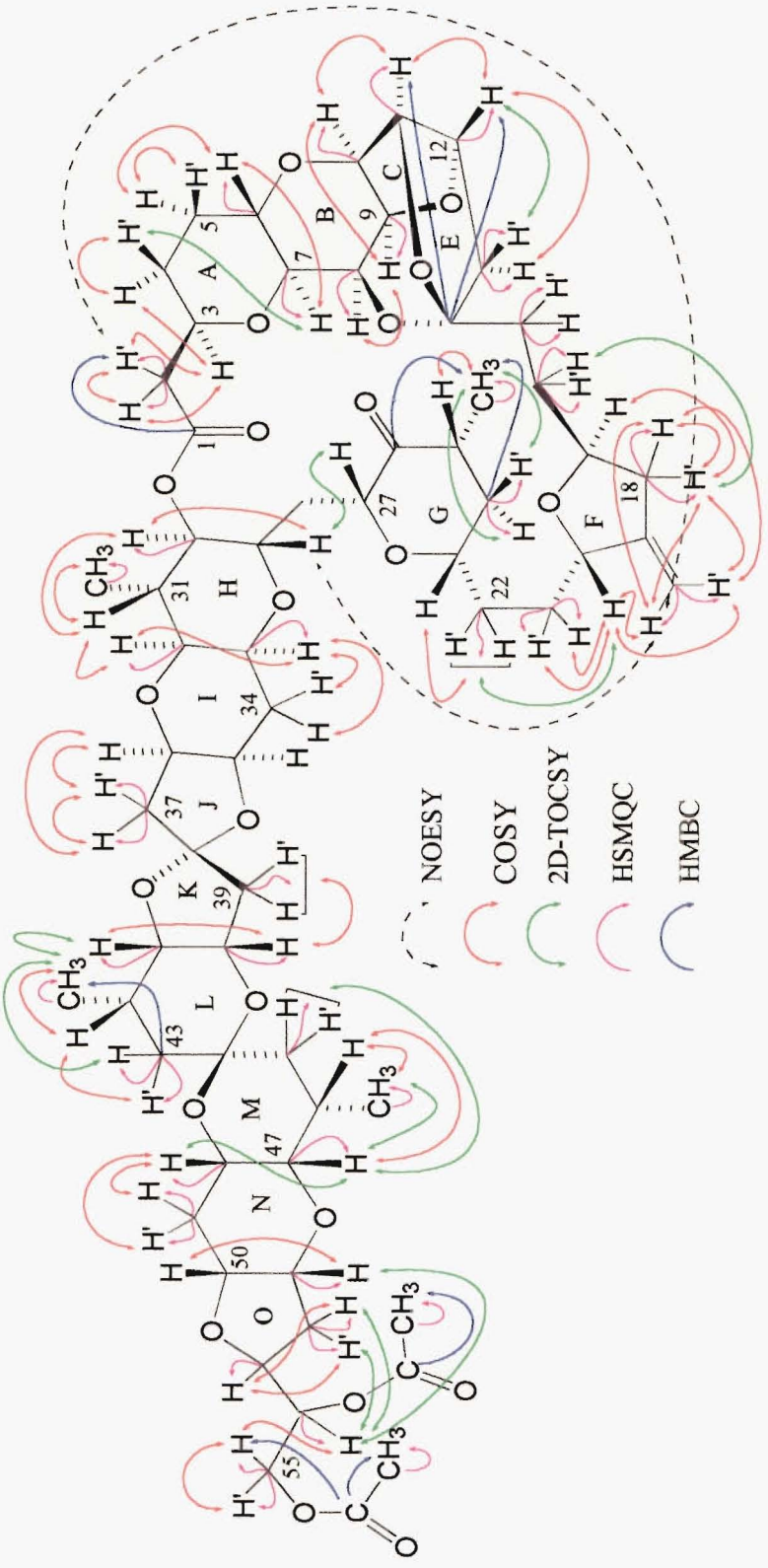
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 5.3.6 ^{13}C NMR Data for Homohalichondrin B Diacetate 26-Ketone (**5.8**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	170.7	C22	30.9	C-CH_3-42	25.7
C2	40.0	C23		C-CH_3-42	17.4
C3		C24	41.8	C43	37.2
C4	30.6	C-CH_3-25	42.4	C44	96.6
C5	30.0	C-CH_3-25	14.7	C45	37.0
C6	68.2	26C=O	209.3	C-CH_3-46	28.8
C7	77.6	C27	77.6	C-CH_3-46	16.8
C8	74.4	C28	33.2	C47	73.0
C9	73.8	C29	71.1	C48	63.6
C10	76.5	C30	76.2	C49	31.2
C11	82.4	C-CH_3-31	36.2	C50	
C12	81.2	C-CH_3-31	14.4	C51	76.2
C13	48.2	C32	78.2	C52	36.6
C14	109.9	C33	66.3	C53	76.2
C15	34.7	C34	29.2	C54	72.3
C16	27.9	C35		C55	63.8
C17		C36		54O$COCH_3$	170.6
C18	38.5	C37	43.3	54OCOCH_3	21.0
19C=CH_2	151.3	C38	112.3	55O$COCH_3$	170.7
19C=CH_2	104.9	C39	42.4	55OCOCH_3	20.8
C20	75.0	C40	70.8		
C21	30.3	C41	79.4		

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, and referenced to $CHCl_3$, δ_C 77.0 ppm.

Figure 5.3.6 Homohalichondrin B Diacetate 26-Ketone – Important NMR Correlations

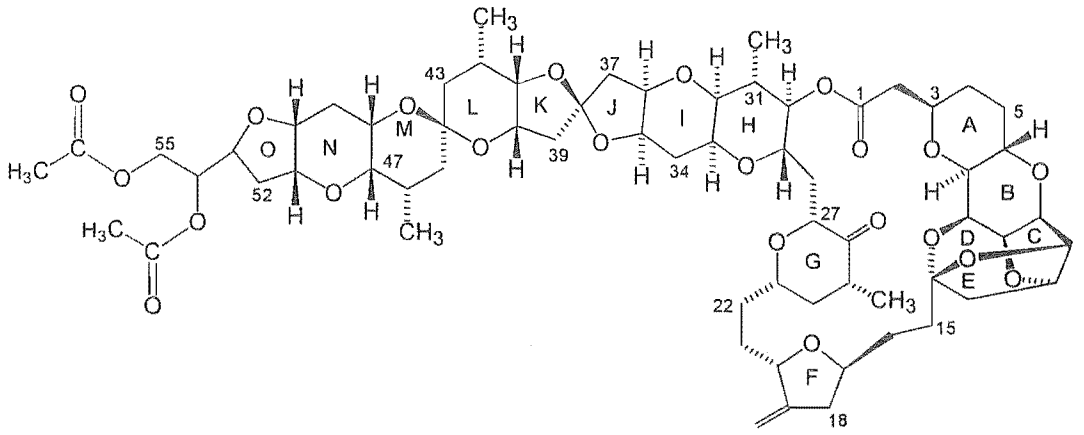


The ^{13}C NMR spectrum contained a new resonance at δ_C 209.3, characteristic of a carbonyl resonance. A correlation between this resonance and the CH_3-25 resonance in the HMBC spectrum of **5.8** confirmed the assignment of this resonance to the C26 carbon. HMBC correlations from the CH_3-25 resonance were also seen to the C24 and C25 resonances. A new HSMQC correlation between δ_C 77.6 and δ_H 3.74 was assigned to C27 and H27 respectively. The assignment of H27 was confirmed by the observation of a TOCSY correlation between this resonance and the H29 resonance (δ_H 4.05). It was established that the connectivity of the remainder of the molecule was identical to that of homohalichondrin B (**1.25**).

The molecular formula of **5.8** was determined as $C_{64}H_{88}O_{22}$ by HRFABMS. This is consistent with the expected loss of CH_4O for diol cleavage.

5.3.7 Characterisation of 5.10

The less polar ketone **5.10** was found to be isobaric with **5.8** by HRFABMS. A comparison of the ^1H NMR spectrum of **5.10** (Figure 5.3.7) with that of **5.8** revealed two major differences. The H32 resonance had shifted upfield δ_H 0.05 to overlap with the H47 resonance, and the broad singlet H41 resonance had shifted downfield from δ_H 3.59. These changes in chemical shift were reminiscent of those observed previously for the homohalichondrin B epimer **2.3**.[95] COSY, 2D-TOCSY (mixing time 100 ms and 120 ms) and HSMQC experiments were run on **5.10**. The partially assigned ^1H and ^{13}C NMR data are listed in Table 5.3.7 and Table 5.3.8. Important NMR correlation are shown in Figure 5.3.8.



38-*epi*-homohalichondrin B diacetate 26-ketone (**5.10**)

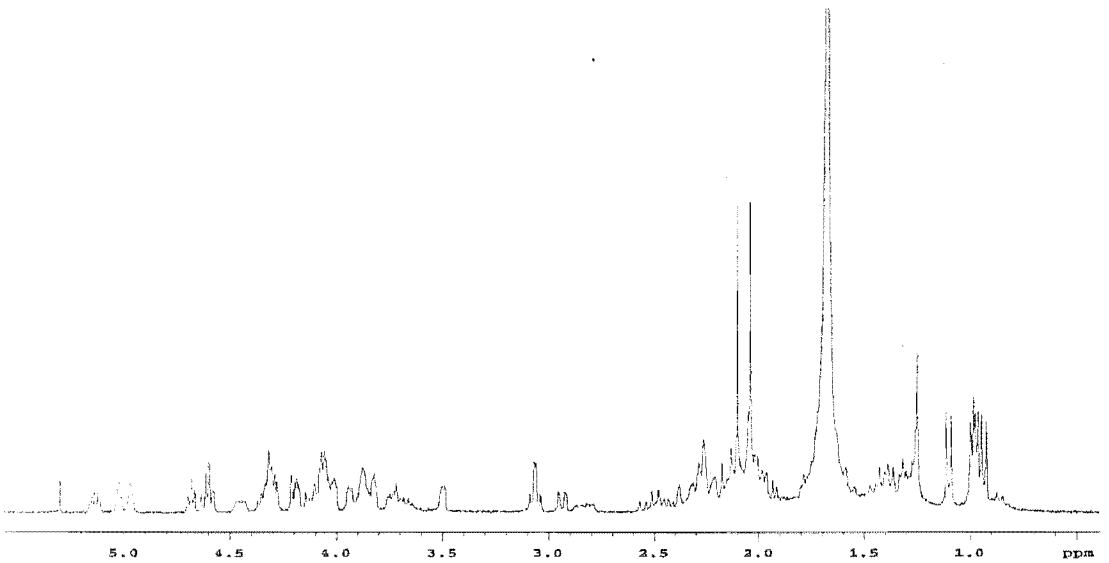


Figure 5.3.7 ^{1}H NMR Spectrum of 38-*epi*-Homohalichondrin B 26-Ketone (**5.10**)

Table 5.3.7 ^{1}H NMR Data for 38-*epi*-Homohalichondrin B Diacetate 26-Ketone (**5.10**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H21**	1.50	**H40**	3.92
H2'	2.51	**H21'**	1.86	**H41**	3.80
H3	3.87	**H22**	1.67	**H42**	2.31
H4	1.40	**H22'**		**CH_3-42**	0.97
H4'	1.67	**H23**	3.83	**H43**	1.30
H5	1.38	**H24**	1.60	**H43'**	1.42
H5'	2.09	**H24'**	2.01	**H45**	1.28
H6	4.29	**H25**	2.45	**H45'**	1.40
H7	2.94	**CH_3-25**	1.10	**H46**	2.07
H8	4.34	**H27**	3.74	**CH_3-46**	0.93
H9	4.06	**H28**		**H47**	3.06
H10	4.18	**H28'**		**H48**	3.50
H11	4.60	**H29**	4.04	**H49**	1.75
H12	4.68	**H30**	4.60	**H49'**	2.17
H13	1.94	**H31**	2.02	**H50**	3.87
H13'	2.16	**CH_3-31**	0.99	**H51**	4.00
H15		**H32**	3.07	**H52**	1.74
H15'		**H33**	3.69	**H52'**	1.97
H16		**H34**	2.06	**H53**	4.31
H16'		**H34'**		**H54**	5.14
H17	4.10	**H35**		**H55**	4.18
H18	2.25	**H36**		**H55'**	4.28
H18'	2.84	**H37**		**54OCOCH$_3$**	2.10
19=CH_2	4.97	**H37'**		**55OCOCH$_3$**	2.04
19'=CH_2	5.02	**H39**	2.03		
H20	4.45	**H39'**	2.29		

[a] The symbol ' represents the less shielded proton of a geminal pair.

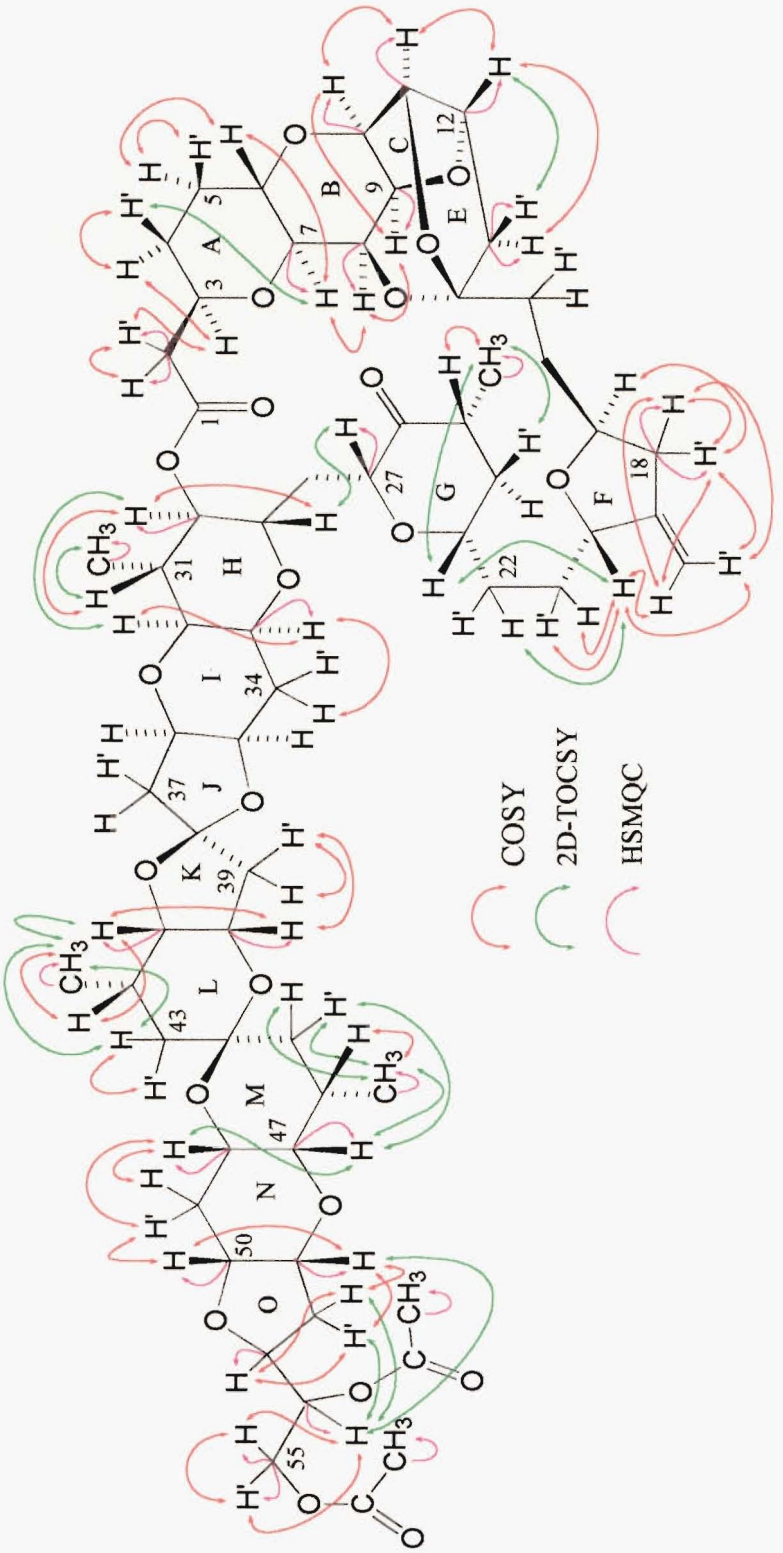
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 5.3.8 ^{13}C NMR Data for 38-*epi*-Homohalichondrin B Diacetate 26-Ketone (**5.10**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1		C22		C-CH_3-42	
C2	40.1	C23		C-CH_3-42	17.6
C3		C24		C43	
C4		C-CH_3-25		C44	
C5		C-CH_3-25	14.8	C45	
C6		26C=O		C-CH_3-46	
C7	77.7	C27	77.5	C-CH_3-46	17.1
C8	74.4	C28		C47	72.9
C9	73.8	C29		C48	63.6
C10	76.5	C30	76.4	C49	
C11	82.2	C-CH_3-31		C50	74.3
C12	81.0	C-CH_3-31	14.4	C51	76.3
C13	48.2	C32		C52	
C14		C33	66.8	C53	76.2
C15		C34		C54	72.2
C16		C35		C55	63.8
C17		C36		54O$COCH_3$	
C18	38.6	C37		54OCOCH_3	21.0
19C=CH_2		C38		55O$COCH_3$	
19C=CH_2		C39		55OCOCH_3	20.7
C20		C40	71.5		
C21		C41	78.8		

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from HSMQC and HMBC spectra at 300 MHz.

Figure 5.3.8 38-*epi*-Homohalichondrin B Diacetate 26-Ketone - Important NMR Correlations



The connectivity of **5.10** was established as being identical to that of the ketone **5.8**. In addition to the aforementioned changes in chemical shift, the H39/H39' methylene protons were no longer equivalent. The chemical shift difference for these protons compared to those of **5.8** is consistent with the formation of the C38 epimer.

5.3.8 Characterisation of 5.9

The molecular formula of **5.9** was determined as $C_{65}H_{92}O_{23}$ by HRFABMS, isobaric to the diol **5.6**. However the ^{1}H NMR spectrum of **5.9** (Figure 5.3.9) was not equivalent to that of **5.6**. Subtle differences in chemical shift were observed in the methyl region of the spectrum, and the region of δ_H 3.7-4.2. The CH_2=19 resonance had shifted upfield slightly, and the H42 resonance was no longer observed at δ_H 3.60. The change in chemical shift of the H42 resonance pointed once again to epimerisation at C38.

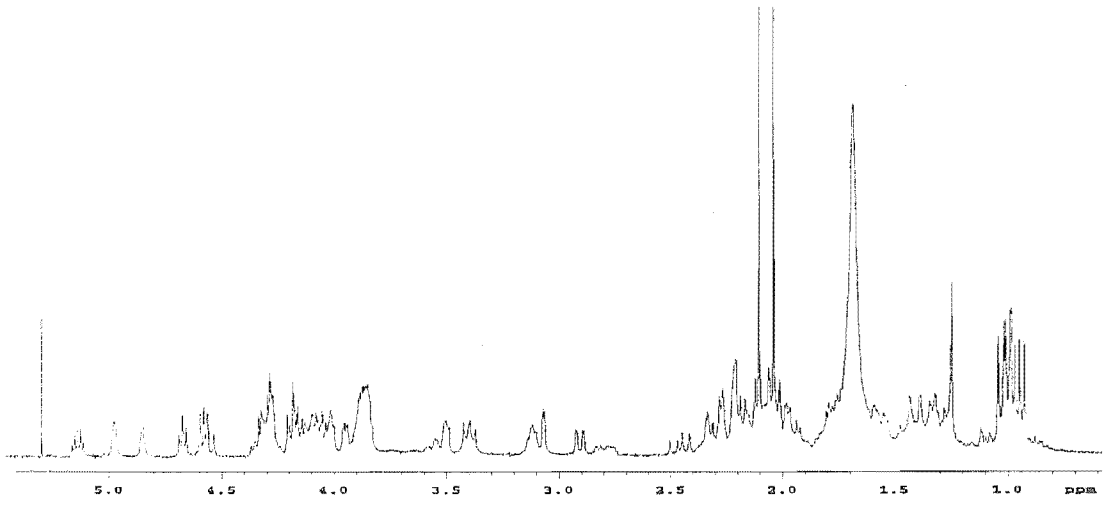


Figure 5.3.9 ^{1}H NMR Spectrum of 38-*epi*-Homohalichondrin B Diacetate 26-Diol (**5.9**)

An array of 2D NMR experiments were performed on **5.9** *viz* COSY, 2D-TOCSY (mixing time 40, 100 and 120 ms) and HSMQC experiments. These data enabled a partial assignment of the ^{1}H NMR and ^{13}C NMR resonances of **5.9** to be achieved. The ^{1}H and ^{13}C NMR data are listed in Table 5.3.9 and Table 5.3.10 and the important NMR correlations are shown in Figure 5.3.10.

Table 5.3.9 ^{1}H NMR Data for 38-*epi*-Homohalichondrin B Diacetate 26-Diol (**5.9**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.30	**H21**		**H39**	2.02
H2'	2.46	**H21'**		**H39'**	2.29
H3	3.85	**H22**		**H40**	3.94
H4	1.31	**H22'**		**H41**	3.86
H4'	1.68	**H23**	3.53	**H42**	2.32
H5	1.30	**H24**	1.09	**CH_3-42**	0.98
H5'	2.06	**H24'**	1.55	**H43**	1.35
H6	4.25	**H25**	1.74	**H43'**	
H7	2.91	**CH_3-25**	1.00	**H45**	1.30
H8	4.31	**26-CH_2**		**H45'**	1.42
H9	4.02	**26'-CH_2**		**H46**	2.06
H10	4.16	**H27**	3.12	**CH_3-46**	0.94
H11	4.58	**H28**	1.77	**H47**	3.07
H12	4.67	**H28'**	2.00	**H48**	3.50
H13	1.94	**H29**	4.11	**H49**	1.75
H13'	2.12	**H30**	4.56	**H49'**	2.14
H15		**H31**	1.98	**H50**	3.88
H15'		**CH_3-31**	1.03	**H51**	4.01
H16	1.39	**H32**	3.40	**H52**	1.74
H16'		**H33**	3.87	**H52'**	1.99
H17	4.08	**H34**		**H53**	4.33
H18	2.24	**H34'**		**H54**	5.14
H18'	2.80	**H35**		**H55**	4.18
19=CH_2	4.85	**H36**		**H55'**	4.29
19'=CH_2	4.98	**H37**		**54OCOCH_3**	2.10
H20	4.29	**H37'**		**55OCOCH_3**	2.04

[a] The symbol ' represents the less shielded proton of a geminal pair.

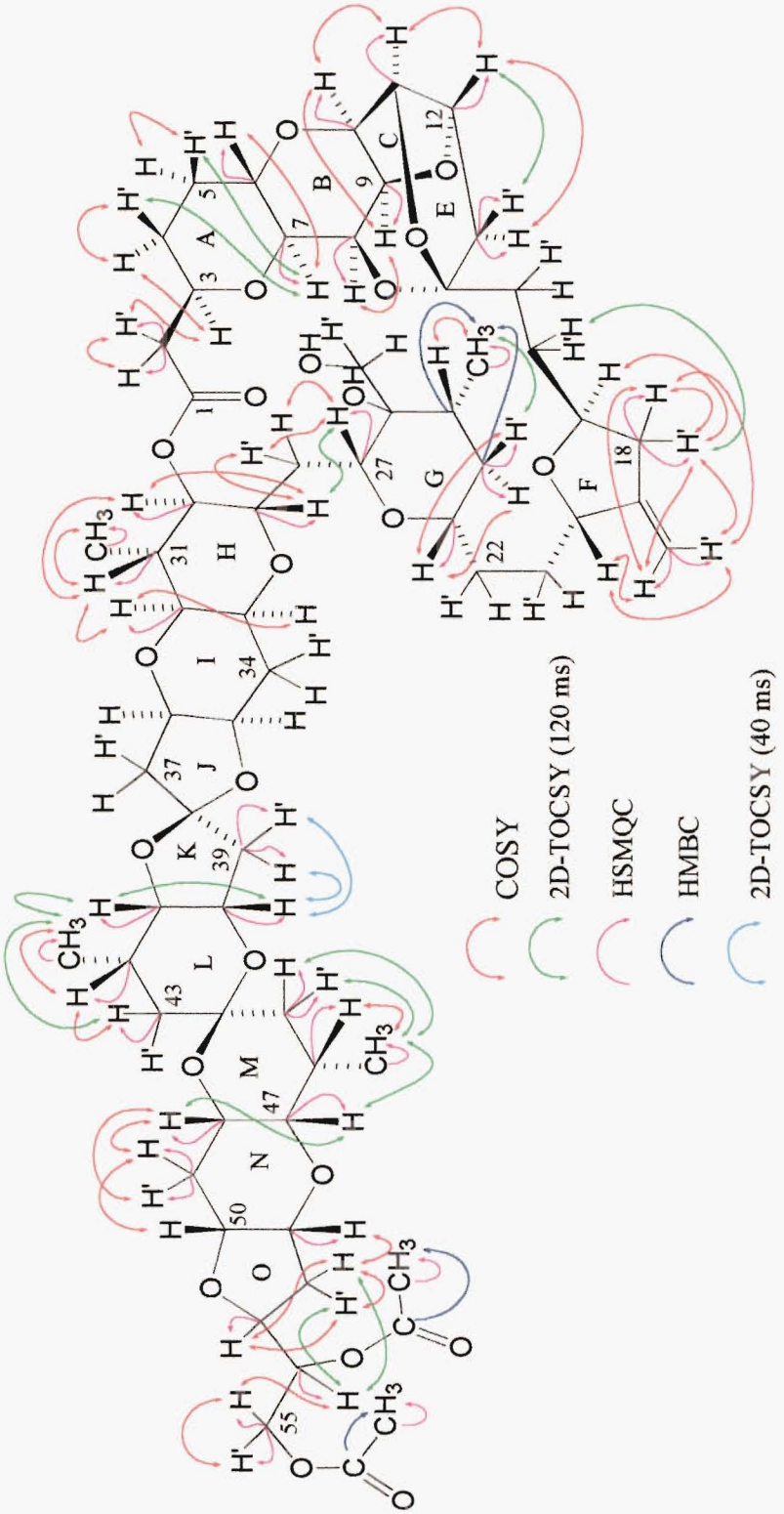
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 5.3.10 ^{13}C NMR Data for 38-*epi*-Homohalichondrin B Diacetate 26-Diol (**5.9**)

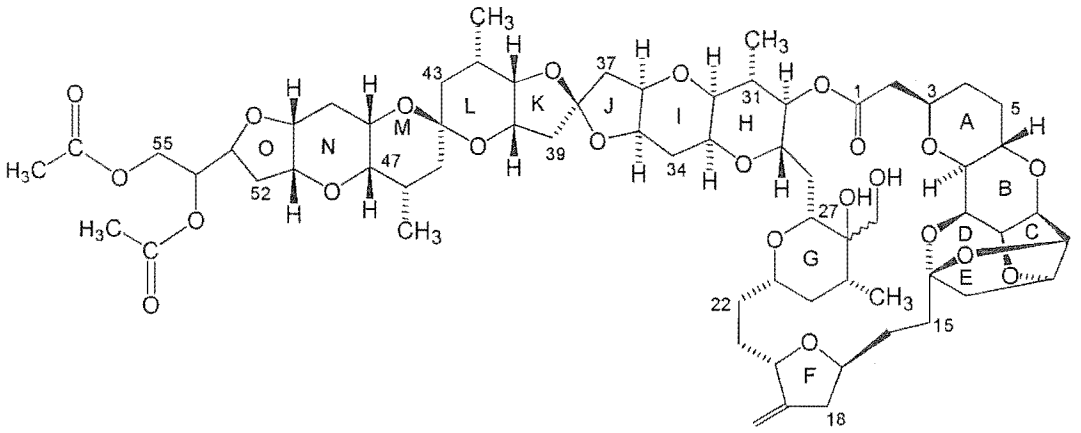
Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1		**C22**		**C41**	78.8
C2	40.2	**C23**	75.5	**C**-CH_3-**42**	25.3
C3		**C24**	38.6	**C**-CH_3-**42**	17.5
C4		**C**-CH_3-**25**	40.8	**C43**	37.0
C5		**C**-CH_3-**25**	14.7	**C44**	
C6	68.1	**26C**-CH_2		**C45**	37.2
C7	77.7	**26C**-CH_2		**C**-CH_3-**46**	28.8
C8	74.1	**C27**	79.3	**C**-CH_3-**46**	17.1
C9	73.8	**C28**		**C47**	72.9
C10	76.6	**C29**	69.6	**C48**	63.5
C11	82.2	**C30**	74.1	**C49**	31.0
C12	81.1	**C**-CH_3-**31**	37.8	**C50**	
C13	48.2	**C**-CH_3-**31**	14.7	**C51**	76.5
C14		**C32**	75.3	**C52**	
C15	34.8	**C33**		**C53**	76.2
C16		**C34**		**C54**	72.4
C17	75.6	**C35**		**C55**	63.8
C18	38.8	**C36**		**54OC**OCH_3	170.6
19C=CH_2		**C37**	44.0	**54OCOC**H_3	20.9
19C=CH_2	103.8	**C38**		**55OC**OCH_3	170.6
C20		**C39**	44.1	**55OCOC**H_3	20.6
C21		**C40**	71.5		

[a] Data recorded at 23°C in $CDCl_3$ with chemical shifts in ppm, assigned from HSMQC and HMBC spectra at 300 MHz.

Figure 5.3.10 38-*epi*-Homohalichondrin B Diacetate 26-Diol – Important NMR Correlations



The limited ^{13}C NMR data obtained for the G-ring are in good agreement with the data for the diol **5.6**. The ^{1}H NMR data, while not identical, are consistent with the presence of a diol at C26. The changes in chemical shift, relative to **5.6**, for the H39, H39', H41 and CH_3-42 resonances are consistent with epimerisation at C38. Further evidence for epimer formation is provided by the retention time of the diol **5.9**, which is similar to that of the ketone **5.8**. Experience with chromatography of halichondrin epimers under reverse phase (C18) conditions has shown that the retention time of the C38 epimer is considerably greater than that of the parent halichondrin (refer Section 2.2).



38-*epi*-homohalichondrin B diacetate 26-diol (**5.9**)

5.3.9 Periodate Cleavage of 5.7

The combined tetraol material (Section 1.3.1 and 1.3.3) was treated with an aqueous solution of sodium periodate for 21 hours before extraction with EtOAc to give the diketone **5.1**. The molecular formula of $C_{63}H_{86}O_{23}$ determined by HRFABMS confirmed diketone formation. The ^{1}H NMR spectrum of **5.1** was comparable to that of an authentic sample.

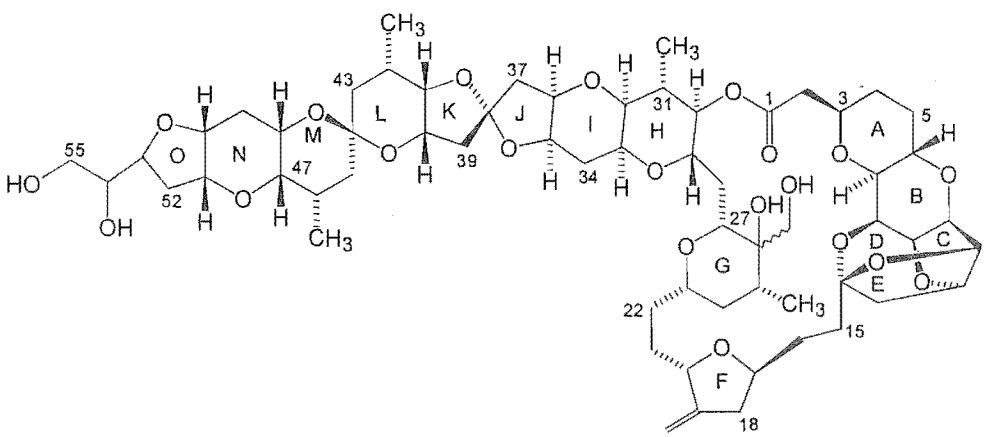
Some of this material was subsequently used for a trial methylenation reaction (Section 5.4.1.2).

5.3.10 Deprotection of 5.6

The deprotection of homohalichondrin B diacetate diol (**5.6**) was undertaken to enable the comparison of activity between the diol **5.6** and the deprotected tetraol.

Diol **5.6** (0.3 mg) was treated with a 4:1 MeOH/H_2O solution of 1% K_2CO_3 for 22 hours. The reaction mixture was diluted with water and the product extracted with EtOAc. A sharp singlet resonance at δ_H 2.04 in the ^1H NMR spectrum of the organic extract indicated incomplete cleavage of the 55-acetate group.

The reaction was repeated, treating a sample of the diol **5.6** with a 4:1 MeOH/H_2O solution of 1% K_2CO_3 for 72 hours at room temperature before extraction with EtOAc. A ^1H NMR spectrum of the product showed that cleavage of the acetate protecting groups was complete. However, two sets of resonances were observed for the H7, CH_2=19 and CH_2=19' protons. A molecular formula of $C_{61}H_{88}O_{21}$ for the major constituent of the product mixture homohalichondrin B 26-diol (**5.11**) was determined by HRFABMS. This is consistent with cleavage of the acetate groups. Other ions were observed in the mass spectrum at 18 mu and 32 mu greater than the parent ion, consistent with cleavage of the lactone to furnish the acid and the methyl ester respectively.



homohalichondrin B 26-diol (**5.11**)

5.3.11 Biological Activity

The biological activity of selected derivatives was evaluated against the P388 murine leukemia cell line, and against the NCI's 60-cell line panel. The results from the screening are shown below in Table 5.3.11, together with the results for the parent homohalichondrin B (**1.25**) and the COMPARE "seed" compound halichondrin B (**1.8**). The tetrahydro derivative has also been included.[95]

Table 5.3.11 *In Vitro* Cytotoxicities of Selected Halichondrins

Compound	**P388 IC_{50} (ng/mL)**	**NCI GI_{50} ($\times 10^{-10}$ M)**	**COMPARE Correlation**
halichondrin B (1.8)	0.78	1.38	1.00
homohalichondrin B (1.25)	0.22	3.16	0.95
homohalichondrin B di-Ac 19,26-tetraol (5.7)	> 12500	85.1	0.46
homohalichondrin B di-Ac 19,26-diketone (5.1)	27.7	49.0	0.64
homohalichondrin B di-Ac 26-diol (5.6)	1414	3890	0.12
homohalichondrin B 26-diol (5.11)	2602	7079	0.08
tetrahydro homohalichondrin B	12.6	362	0.87

These data clearly illustrate the importance of the exocyclic methylenes to the biological activity in the halichondrin series. All of the derivatives described in this chapter exhibit significantly reduced biological activity relative to the parent homohalichondrin B (**1.25**). Polarity also appears to be have a role in the retention of cytotoxicity. Both the diketone **5.1** and the tetrahydro derivative are significantly more cytotoxic than the hydroxy derivatives in the P388 cell line. However, the NCI's 60 cell line panel is more sensitive to the tetraol **5.7**. It is interesting to note that the tetraol **5.7** is more cytotoxic

than the diol **5.6**, which still contains the 19-olefin. This would seem to reflect a change in conformation for the diol, relative to the tetraol, that has a detrimental effect on the activity of the diol **5.6**. However, evidence from the NOESY spectrum of **5.6** indicates that the conformation of the diol in the region of the 19 methylene is similar to that of the parent homohalichondrin B (**1.25**). The characteristic NOESY correlation (across the lactone ring) between the H20 and H2' protons was observed, together with a correlation between the H29 and CH_2-19' protons.

5.4 Radioactive Labelling

The formation of radiolabelled halichondrin B (**1.8**) would provide a means of detection of **1.8** and possibly some of its metabolites in biological systems. This would be useful for pharmacokinetic studies of **1.8**. Radioactive labelling would also be useful for further probing of the mechanism of action of halichondrins in *in vitro* cell studies.

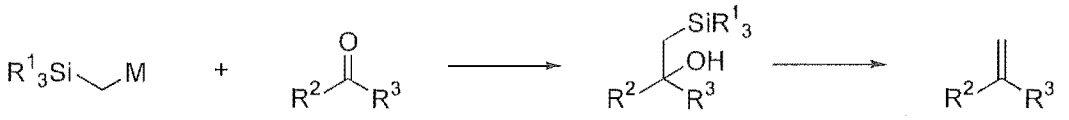
5.4.1 Methylenation

Incorporation of a radioactive label into halichondrin B (**1.8**) requires the reformation of **1.8** from one of its derivatives. The diketone **5.1** was seen as a likely starting material. Any method that was successful in methylenating the homohalichondrin B derivative **5.1** could then be applied to the equivalent halichondrin B derivative. It had already been established that the exocyclic methylenes could not be formed using Wittig chemistry.[95]

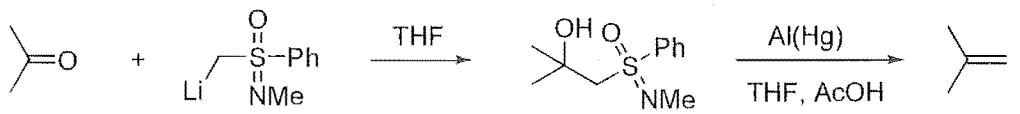
Alternative methods for ketone methylenation were investigated. The addition of a methylene chloride solution of $TiCl_4$ (0.7 parts), to a mixture of CH_2Br_2 (1 part) and zinc dust (3 parts) yields a reagent which smoothly olefinates a variety of ketones.[102] A variation of the original procedure has been used in gibberellin synthesis.[103] The reagent (commonly referred to as 'Lombardo's reagent') is prepared from CH_2Br_2 (1 part), zinc dust (3 parts) and $TiCl_4$ (0.73 parts) in THF at low temperatures and is then allowed to

age at 5°C for three days. The resultant grey slurry reacts smoothly with ketones containing sensitive functionality.[104] The Tebbe reagent[105] is an isolable well characterised compound which also olefinates carbonyl compounds. However, the ability of Tebbe's reagent to methylenate esters and lactones[106] is undesirable in this case. A number of related methylenating reagents have been prepared and tested.[107] One of the main advantages of $CH_2Br_2/Zn/TiCl_4$ is its ready availability.

The conversion of a carbonyl to an alkene can also be achieved by Peterson alkenation[108] (Scheme 5.4.1). This method requires the use of strong base or acid for elimination of the silanolate. The addition of (*N*-methylphenyl sulfonimidoyl)methyllithium followed by reductive elimination (Scheme 5.4.2) is the basis of the Johnson methylenation procedure.[109]



Scheme 5.4.1 Peterson Alkenation



Scheme 5.4.2 Johnson Methylenation

Lombardo's reagent[104] was chosen because it had been used successfully with complex natural products that are acid sensitive, and was selective for ketones over esters. Free hydroxy groups are tolerated by this reagent, and it is suitable for reaction of sterically hindered ketones.[110]

5.4.1.1 Trial Reaction

The conditions for methylenation using Lombardo's reagent were first established using cholestanone as a model. The appearance of a broad resonance at δ_H 4.54 in the ^1H NMR spectrum of the product was consistent with the formation of the CH_2=3 olefin.

Several attempts were required before the reaction was successful. A variation on the Lombardo procedure using catalytic lead[111] was attempted. It was found that the zinc, although previously purified, required purification[112] immediately prior to use.

5.4.1.2 Methylenation of **5.1**

A solution of **5.1** (1.5 mg) in THF was treated with ice cold Lombardo's reagent for 5 minutes. The reaction mixture was neutralised with saturated $NaHCO_3$ and extracted with diethyl ether. Low level resonances corresponding to the H18' proton and the CH_2=19 olefinic protons were observed in the ^1H NMR spectrum of the ether extract. The chemical shift of the olefinic proton resonances (δ_H 4.97, δ_H 5.02) were identical to those observed for the CH_2=19 olefinic resonances in homohalichondrin B diacetate 26-ketone (**5.8**). No evidence was seen for the formation of the CH_2=26 olefin. The poor recovery of material indicated there may be some decomposition occurring. This would most likely be due to the acidic nature of the reaction conditions.

The selective formation of the CH_2=19 olefin is in contrast to the relative reactivity of the olefinic groups. This indicates the conformation of the C1-C30 lactone ring in the diketone derivative **5.1** may be different to that of the parent **1.25**.

5.5 Summary

The EMCH hapten was not prepared because the relationship with Hawaii Biotechnology Group Inc had fallen through, and as a result there was uncertainty as to the future direction of the immunoassay development. It was found that the ketone **5.8** deteriorated over time, and consequently there was insufficient material to revisit this work. However, methodology for the selective derivatisation of the halichondrin C26 exocyclic methylene has been established. Reaction of the ketone **5.8** with EMCH should provide a hapten that would elicit antibodies recognising the terminus of the

halichondrin skeleton. This would allow the development of an immunoassay with the ability to distinguish between members of the B-series halichondrins.

Chapter 6

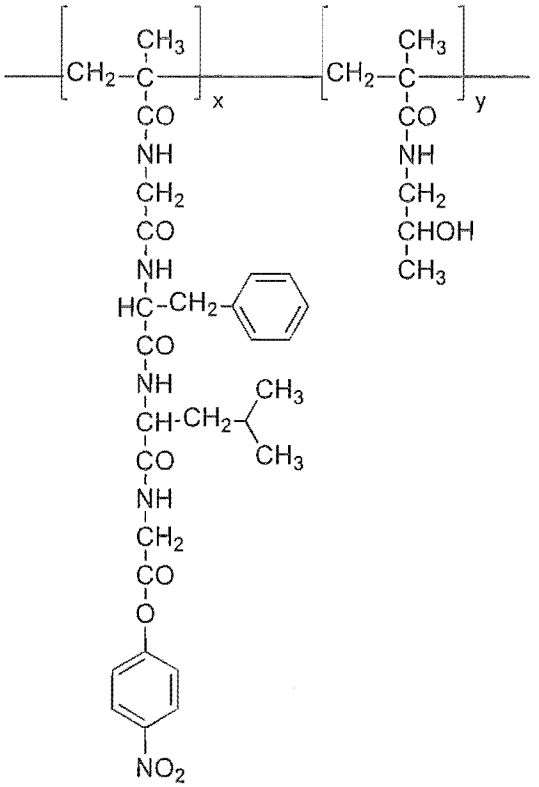
Polymer Drug Conjugates

6.1 Introduction

A general overview of polymer therapeutics is presented in Section 1.4. The synthesis of a polymer drug construct incorporating a halichondrin was perceived as a means of enhancing the future prospects of the clinical development of the halichondrins. Attachment of a halichondrin to a polymer should improve the pharmacokinetic profile, and enhance the selectivity of the drug.

The polymer therapeutics work was undertaken in collaboration with Ruth Duncan's group at the School of Pharmacy, University of London. This group provided the polymer precursor used in the work described in this chapter.

The polymer precursor (**6.1**) is constructed by the copolymerisation of *N*-(2-hydroxypropyl)methacrylamide with methacryloylglycylphenylalanylleucylglycyl *p*-nitrophenyl ester (MA-Gly-Phe-Leu-Gly-ONp) using the method described by Kopecek.[113] The estimated nitrophenyl ester content of the polymeric precursor is 3.98 mol%.[114]



polymer precursor (**6.1**)

The optimal number and combination of amino acids in the spacer required for controlled cleavage by lysosomal enzymes was determined as Gly-Phe-Leu-Gly.[115] Attachment of the drug to the linker by displacement of the *p*-nitrophenoxide (ONp) can be achieved through a number of functional groups including esters, amides, urethanes, hydrazones and thioethers.[116] Controlled release of the drug at the site of action requires this linkage to be stable in the blood stream.

The formation of an ester linkage through the terminal hydroxy group of norhomohalichondrin B (**4.17**) would be a simple means of attaching a halichondrin to a polymer precursor. However, ester linkages have been found to be unstable *in vivo* in some cases, leading to uncontrolled release of the drug.[83] The attachment of norhomohalichondrin B aldehyde (**4.13**) to the polymer *via* a hydrazone linkage would use chemistry already shown to be successful in the attachment of the hydrazine linker EMCH (Section 4.5). However, the UV-active ONp is displaced from the polymer

precursor with hydrazine prior to the linking of norhomohalichondrin B aldehyde (**4.13**). This means that it would not be possible to quantify the amount of halichondrin bound to the polymer. Aminolysis of the ONp ester with an alkyl amine was chosen as the preferred method of halichondrin attachment. Spectroscopic measurement of the free ONp released upon displacement by the halichondrin could be used to quantify the amount of halichondrin attached.

The attachment of halichondrin to polymer through an amide bond required the formation of an aminohalichondrin. Reductive amination of the aldehyde **4.13** with an amine was attempted, as a one-pot route to the desired product. An alternative route *via* an azide intermediate was also investigated.

6.2 Reductive Amination

6.2.1 Trial Reactions

Cholestan-3-one was used as a model compound to establish conditions required for reductive amination because of its size, multiringed structure and availability. Sodium cyanoborohydride ($NaCNBH_3$) was selected as the reducing agent because reduction of aldehydes by this reagent at pH 6-7 in MeOH is negligible.[117]

In the initial attempt phenethylamine hydrochloride, sodium acetate and acetic acid were added to cholestanone and stirred for 30 minutes before the addition of $NaCNBH_3$. The reaction was stirred for 64 hours at room temperature. The solvent was evaporated and the CH_2Cl_2 soluble material was absorbed onto a normal phase (DIOL) cartridge. The product was eluted with CH_2Cl_2/EtOAc (1:1) and CH_2Cl_2. The presence of a multiplet at *ca* δ_H 2.97 in the ^1H NMR spectrum of the product, together with the molecular formula of $C_{35}H_{57}N$, determined by HRFABMS, confirmed reductive amination had occurred.

To ascertain the feasibility of generating a primary amine, cholestanone, ammonium acetate and acetic acid were stirred at room temperature before the addition of $NaCNBH_3$. After stirring the reaction for a further 68 hours the solvent was evaporated. The residue was purified by reverse phase (C18) chromatography. Visualisation of the TLC of the products with Dragendorff's[125] spray reagent confirmed the presence of a primary amine, consistent with the reductive amination of cholestanone with ammonium acetate.

6.2.2 Halichondrin Reactions

Norhomohalichondrin B aldehyde (**4.13**, 0.5 mg), ammonium acetate and acetic acid in MeOH were stirred together for 15 minutes before the addition of $NaCNBH_3$. The reaction was stirred at room temperature for 65 hours. The complex nature of the methyl region in the ^{1}H NMR spectrum of the reaction mixture indicated that more than one component was present. The mixture was separated by reverse phase (C18) HPLC using 55% CH_3CN/H_2O as the mobile phase into five fractions. There was insufficient material (< 0.1 mg) for investigation of these fractions by ^{1}H NMR spectroscopy.

The use of activated 3Å molecular sieves can improve the yield of a reductive amination reaction.[117] The reductive amination of **4.13** with ammonium acetate was repeated, with the inclusion of crushed 3Å molecular sieves. The complexity of the reaction mixture again was indicated by the presence of five halichondrin-like peaks in the HPLC chromatogram. The scale of the reaction (0.6 mg) meant that separation of these components was not productive.

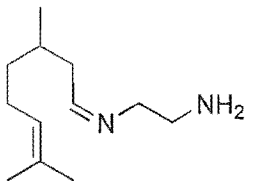
Owing to the difficulties experienced with the reductive amination of norhomohalichondrin B aldehyde (**4.13**), it was decided that further trial reactions would be attempted using an aldehyde (citronellal) rather than a ketone (cholestanone) as a model compound.

6.2.3 Further Trial Reactions

Citronellal was obtained by oxidising citronellol using Dess-Martin periodinane.[118] Reaction of citronellal with ammonium acetate was unsuccessful. It was suggested[119] that ethylenediamine hydrochloride would be a more suitable amine, as it has a pKa in the desired range of 6-8.

A solution of ethylenediamine hydrochloride (2.7 M) in MeOH was made by combining equimolar amounts of ethylenediamine and ethylenediamine dihydrochloride in MeOH (10 mL).

Ethylenediamine hydrochloride and citronellal were stirred with $NaCNBH_3$ until TLC indicated the reaction was complete (17 h). The reaction mixture was made alkaline by the addition of Na_2CO_3 before extraction with EtOAc. Analysis of the extract by GCMS indicated that the major component (> 80%) was the reductive amination product **6.2**. A minor component was identified as the dimer of **6.2**.



6.2

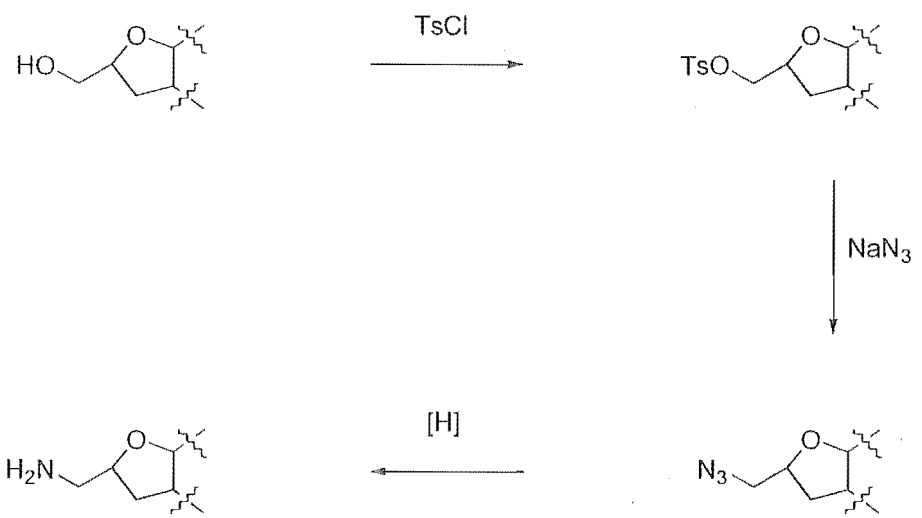
6.2.4 Further Halichondrin Reactions

Norhomohalichondrin B aldehyde (**4.13**, 1.0 mg) and ethylenediamine hydrochloride were stirred in MeOH for 30 minutes before the addition of $NaCNBH_3$. The reaction was stirred for 65 hours at room temperature. The lack of an aldehyde proton resonance in the ^1H NMR spectrum of the reaction mixture indicated that a reaction had occurred. Additional resonances in the region of δ_H 2.5-3.0 were observed. The FABMS spectrum contained a number of seemingly unrelated high mass peaks (m/z 1140-1280). There

was no evidence for production of the desired diamine, nor was there evidence of reduction of **4.13** to the alcohol **4.17**.

Norhomohalichondrin B aldehyde (**4.13**, 0.5 mg) in MeOH was treated with $NaCNBH_3$ for 65 hours at room temperature after which time the integral of the aldehyde proton resonance (δ_H 9.69) had reduced to 20% of the original. LRFABMS ions for MNa^+, MK^+ and MCs^+ for the alcohol **4.17** confirmed that partial reduction of the aldehyde **4.13** had taken place. MNa^+ and MCs^+ ions for the aldehyde **4.13** were also observed.

Owing to the lack of progress with reductive amination of norhomohalichondrin B aldehyde (**4.13**), alternative routes to the halichondrin amine were considered. The azide route (Scheme 6.2.1) utilised simple chemical transformations and did not require the use of acid. Although it was envisaged that the reduction of the azide to the amine may cause problems, a number of methods for conversion of an azide to an amine under relatively neutral conditions exist.[120]



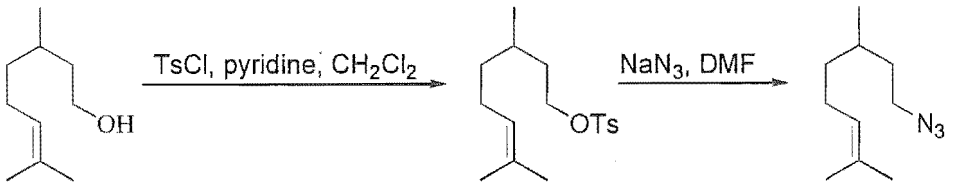
Scheme 6.2.1 An Overview of the Proposed Azide Route

6.3 The Azide Route

6.3.1 Trial Reactions

Citronellol (**6.3**) was used as the starting material for a series of reactions to establish a route to the amine. The stability of the olefinic functionality to the reaction sequence was an important consideration.

Treatment of **6.3** with *p*-toluenesulfonyl chloride yielded the tosylate **6.4**. The shift of the α-methylene resonance to a less shielded position (δ_H 4.05) relative to the alcohol (δ_H 3.63) was consistent with tosylate formation, along with the presence of a methyl resonance at δ_H 2.45. The azide **6.5** was formed by reaction of **6.4** with NaN_3. The expected shift in the α-methylene resonance to a more shielded position (δ_H 3.28) was observed.



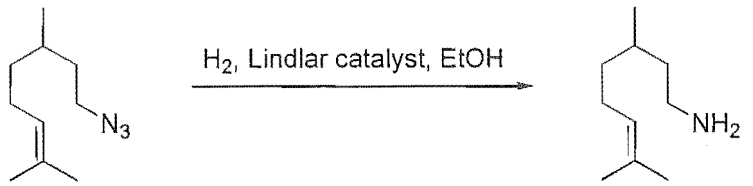
6.3 **6.4** **6.5**

Initial attempts to reduce the azide used Staudinger methodology.[121] The removal of excess triphenylphosphine from the reaction mixture proved to be an insurmountable problem, not helped by the volatility of the citronellamine. It was never established if any product was formed. Reduction using phase transfer catalysis[122] (ammonium formate, Pd/C, 23 hours, 20°C) was also unsuccessful, with only starting material being recovered.

Tosylation of **6.3** was repeated on a larger scale (7.2 mmol) to enable a more accurate mass balance to be achieved following reduction of the azide. A solution of **6.3**, DMAP and diisopropylethylamine in CH_2Cl_2 was cooled to 0°C before adding TsCl. The ^1H NMR spectrum of the worked up extract contained two sets of olefinic and α-methylene

resonances. The relative integrals of the α-methylene resonances indicated that 55% of the mixture was tosylate. The chemical shift of the α-methylene resonance of the other component (δ_H 3.68) was consistent[123] with substitution of the tosylate by chloride. This was subsequently confirmed by EIMS. The mixture was treated with NaN_3 to give a mixture of the azide **6.5** and the chloride. Pure azide was obtained by flash chromatography on silica, eluting with petroleum ether.

Selective hydrogenation of the azide **6.5** was achieved by the use of Lindlar catalyst.[124] The retention of the olefin was confirmed by the presence of a multiplet at δ_H 5.07 in the ^{1}H NMR spectrum of the product. GCMS was used to ascertain that the amine **6.6** was 95% pure. The molecular formula of $C_{10}H_{21}N$, determined by HREIMS, was consistent with amine formation.



6.5 **6.6**

6.3.2 Halichondrin Reactions

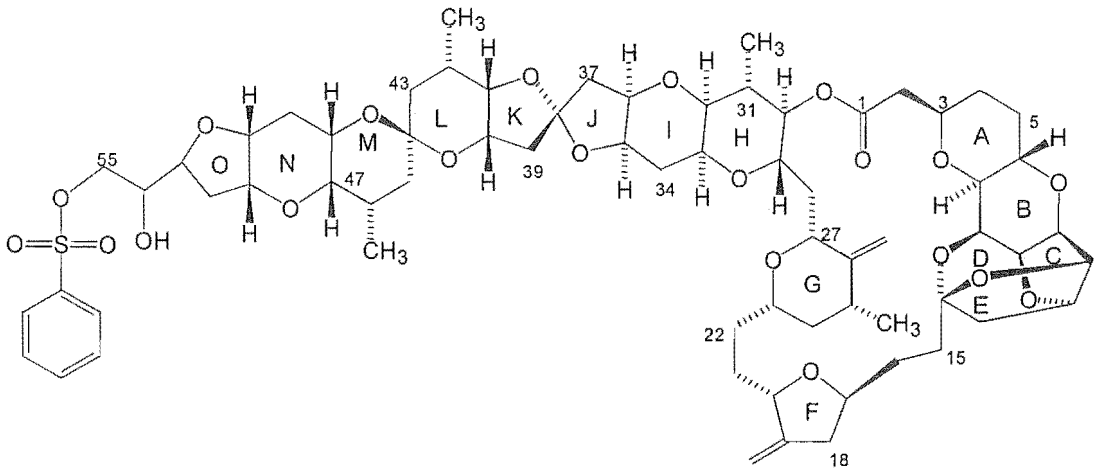
6.3.2.1 Attempted Tosylation of Norhomohalichondrin B (**4.17**)

p-Toluenesulfonyl chloride was added to a solution of norhomohalichondrin B (**4.17**, 0.9 mg), DMAP and diisopropylethylamine in CH_2Cl_2 at 0°C. The reaction was allowed to warm to room temperature and stirred for 2 hours. The ^{1}H NMR spectrum of the EtOAc extract showed that no reaction had occurred. The reaction was repeated, doubling the quantities of reagents used. Once again, the ^{1}H NMR spectrum of the EtOAc extract was comparable to that of the starting material. A third attempt was made, using a large excess (*ca* twenty-fold) of TsCl. The ^{1}H NMR spectrum of the organic extract was dominated by an unidentified contaminant. Low-level halichondrin-

type resonances were observed. TLC (DIOL, 4% MeOH/CH_2Cl_2) indicated that starting material was present in the contaminated mixture.

6.3.2.2 Tosylation of Homohalichondrin B (**1.25**)

Tosyl chloride was added to a solution of homohalichondrin B (**1.25**, 1.5 mg), DMAP and diisopropylethylamine in CH_2Cl_2 at 0°C. The reaction was allowed to warm to room temperature and stirred for 2 hours. The observation of a shoulder on the H47 resonance indicated that reaction had occurred. However the starting material was largely intact. The reaction was repeated and stirred for 18 hours. The absence of the characteristic H55/55' resonance at δ_H 3.69 was consistent with tosylation of the 55 hydroxy group. The H47 resonance had shifted to a more shielded position (δ_H 3.05) relative to that in homohalichondrin B. The molecular formula of $C_{68}H_{92}O_{21}S$ determined by HRFABMS indicated that the monotosylate of homohalichondrin B had been formed.



homohalichondrin B 55-tosylate (**6.7**)

6.3.2.3 Tosylation of Norhomohalichondrin B (**4.17**)

The reaction conditions used for the tosylation of homohalichondrin B (**1.25**) were applied to norhomohalichondrin B (**4.17**). TsCl in CH_2Cl_2 was added to an ice-cold solution of **4.17** (0.5 mg), diisopropylethylamine and DMAP in CH_2Cl_2. The reaction

was allowed to warm to room temperature and stirred for 17 hours. The H54 and H54' resonances of the alcohol **4.17** were not observed in the ^{1}H NMR spectrum of the EtOAc extract, indicating that reaction had occurred. A small amount of TsCl was still present. Changes to resonances in the region of δ_H 3.45-4.15 relative to those of **4.17** were observed. The characterisation of norhomohalichondrin B tosylate (**6.8**) is described in Section 6.3.2.6.

6.3.2.4 Reaction of Norhomohalichondrin B Tosylate (**6.8**) with Sodium Azide

Norhomohalichondrin B tosylate (**6.8**, 0.3 mg) was treated with an excess of NaN_3 in DMF at 40°C for 18 hours. Analysis of the EtOAc extract by reverse phase (C18) HPLC, using a mobile phase of 70% CH_3CN/H_2O, showed that two major components were present. The tosylate **6.8** eluted at 1080 s, after a component of similar polarity at 996 s. The UV spectrum of the tosylate contained absorbance maxima at λ_{194} and λ_{226}. The UV spectrum of the component at 996 s contained an end absorption maxima only (λ_{194}). This was consistent with substitution of the tosylate by azide. The ratio of azide to tosylate was estimated to be 4:1 from the refractive index-detected chromatogram.

6.3.2.5 Attempted Hydrogenation

The azide/tosylate mixture was used without further purification in an effort to establish if selective reduction of the azide in the presence of the exocyclic methylenes of the halichondrin skeleton was possible. It was anticipated that this hydrogenation could be accomplished successfully based on the selective reduction of the azide in the model compound **6.5** (see Section 6.3.2.3).

Hydrogenation of the azide/tosylate mixture was carried out with Lindlar catalyst in ethanol at room temperature and atmospheric pressure for 5 hours. The reaction was filtered through Celite to remove the catalyst and the solvent evaporated. The ^{1}H NMR spectrum of the residue was identical to that of the starting material.

Hydrogenation was repeated using a longer reaction time of 48 hours. The exocyclic methylenes were retained under these conditions, but it was not possible to ascertain if any reduction of the azide had occurred.

The apparent robustness of the exocyclic methylenes to hydrogenation with Lindlar catalyst was promising enough to commit further halichondrin material to the reaction. A "large scale" reaction was undertaken with the periodate cleavage of 44 mg of homohalichondrin B (**1.25**). Representative examples of this reaction and the subsequent reduction of the aldehyde **4.13** with sodium borohydride in IPA are described in Section 4.4 and 4.6. The ^{1}H NMR spectra obtained for the reaction products were comparable to those obtained previously.

6.3.2.6 Formation of Norhomohalichondrin B Tosylate (**6.8**)

Norhomohalichondrin B (**4.17**, 19.1 mg), diisopropylethylamine and DMAP were cooled to 0°C in CH_2Cl_2. TsCl in CH_2Cl_2 was added dropwise. The reaction was allowed to warm to room temperature and stirred for 14 hours. The ^{1}H NMR spectrum of the organic extract (Figure 6.3.1) was comparable to that obtained previously for the tosylate **6.8** (Section 6.3.2.3).

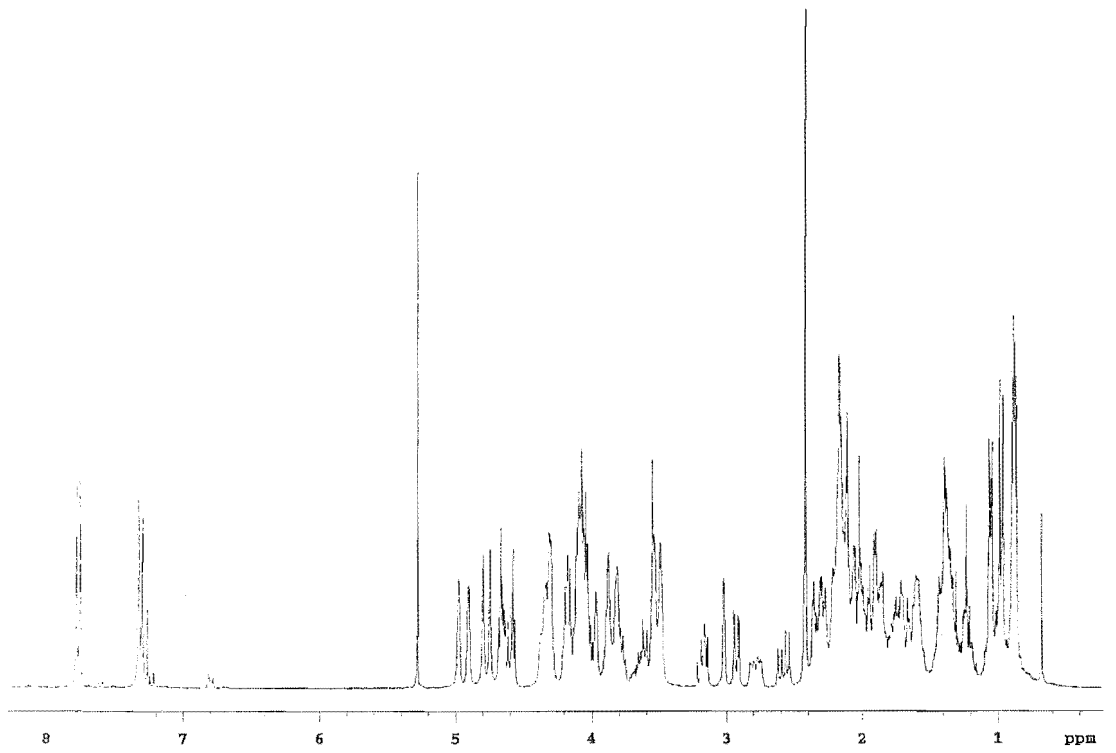


Figure 6.3.1 ^{1}H NMR Spectrum of Norhomohalichondrin B Tosylate (**6.8**)

A full range of NMR experiments were performed on **6.8** *viz* COSY, 1D-TOCSY, 2D-TOCSY (mixing time 100 ms), HSQC, HMBC and APT (Figure 6.3.2) experiments. These data enabled the complete assignment of the ^{1}H NMR and ^{13}C NMR resonances of **6.8** to be achieved. The ^{1}H and ^{13}C NMR data are listed in Table 6.3.1 and Table 6.3.2, and the important correlations from the 2D experiments are shown in Figure 6.3.3

Table 6.3.1 ^{1}H NMR Data for Norhomohalichondrin B Tosylate (**6.8**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.33	**H21**	1.38	**H39**	2.16
H2'	2.58	**H21'**	1.87	**H39'**	2.16
H3	3.87	**H22**	1.59	**H40**	3.87
H4	1.36	**H22'**	1.59	**H41**	3.54
H4'	1.72	**H23**	3.52	**H42**	2.30
H5	1.36	**H24**	1.02	**CH_3-42**	0.89
H5'	2.08	**H24'**	1.67	**H43**	1.22
H6	4.31	**H25**	2.19	**H43'**	1.37
H7	2.93	**CH_3-25**	1.05	**H45**	1.37
H8	4.30	**26=CH_2**	4.75	**H45'**	1.37
H9	4.02	**26'=CH_2**	4.80	**H46**	2.13
H10	4.16	**H27**	3.52	**CH_3-46**	0.86
H11	4.58	**H28**	1.93	**H47**	3.02
H12	4.67	**H28'**	1.99	**H48**	3.48
H13	1.92	**H29**	4.18	**H49**	1.74
H13'	2.13	**H30**	4.64	**H49'**	2.08
H15	1.59	**H31**	2.00	**H50**	3.81
H15'	2.16	**CH_3-31**	0.98	**H51**	3.96
H16	1.41	**H32**	3.16	**H52**	1.87
H16'	2.14	**H33**	3.79	**H52'**	2.02
H17	4.08	**H34**	1.77	**H53**	4.32
H18	2.24	**H34'**	2.13	**H54**	4.02
H18'	2.78	**H35**	4.09	**H54'**	4.08
19=CH_2	4.91	**H36**	4.08	**H56**	7.77
19'=CH_2	4.98	**H37**	1.88	**H57**	7.32
H20	4.35	**H37'**	2.32	**H59**	2.40

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 6.3.2 ^{13}C NMR Data for Norhomohalichondrin B Tosylate (**6.8**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	170.9	**C22**	32.1	**C41**	79.3
C2	40.4	**C23**	74.8	**C-CH_3-42**	25.7
C3	73.8	**C24**	43.4	**C-CH_3-42**	17.7
C4	30.7	**C-CH_3-25**	35.9	**C43**	36.8
C5	30.1	**C-CH_3-25**	18.0	**C44**	96.5
C6	68.2	**26C=CH_2**	151.6	**C45**	36.8
C7	77.6	**26C=CH_2**	104.0	**C-CH_3-46**	28.7
C8	74.3	**C27**	73.6	**C-CH_3-46**	17.1
C9	73.4	**C28**	36.7	**C47**	73.1
C10	76.5	**C29**	71.1	**C48**	63.5
C11	82.1	**C30**	76.9	**C49**	31.1
C12	81.0	**C-CH_3-31**	36.6	**C50**	74.4
C13	48.3	**C-CH_3-31**	15.1	**C51**	76.6
C14	109.9	**C32**	77.5	**C52**	36.7
C15	34.4	**C33**	66.5	**C53**	75.1
C16	28.2	**C34**	29.1	**C54**	71.5
C17	75.3	**C35**	75.1	**C55**	132.9
C18	38.7	**C36**	76.2	**C56**	127.8
19C=CH_2	151.4	**C37**	43.5	**C57**	129.6
19C=CH_2	104.4	**C38**	112.2	**C58**	144.5
C20	75.3	**C39**	42.6	**C59**	21.7
C21	29.4	**C40**	70.7		

[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

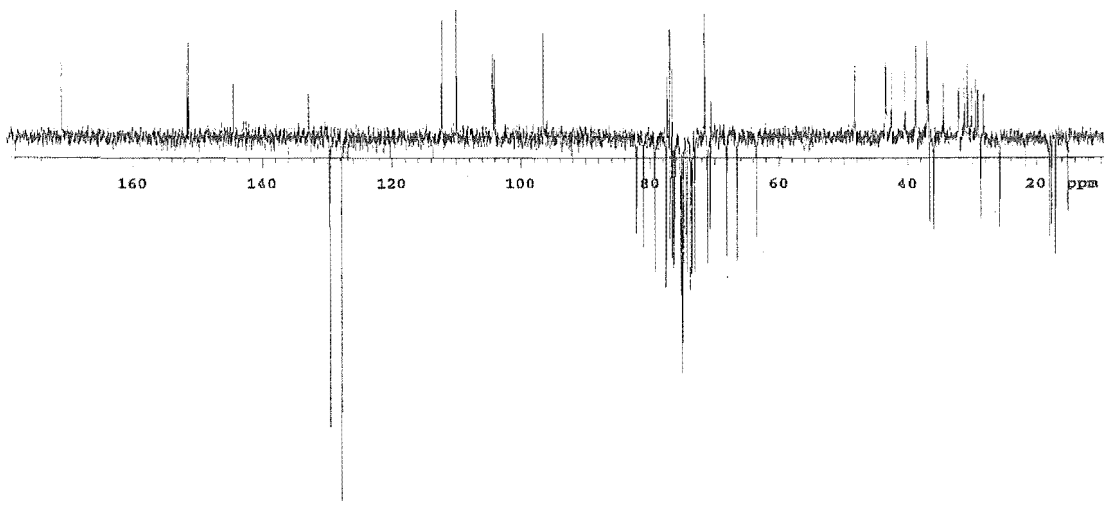
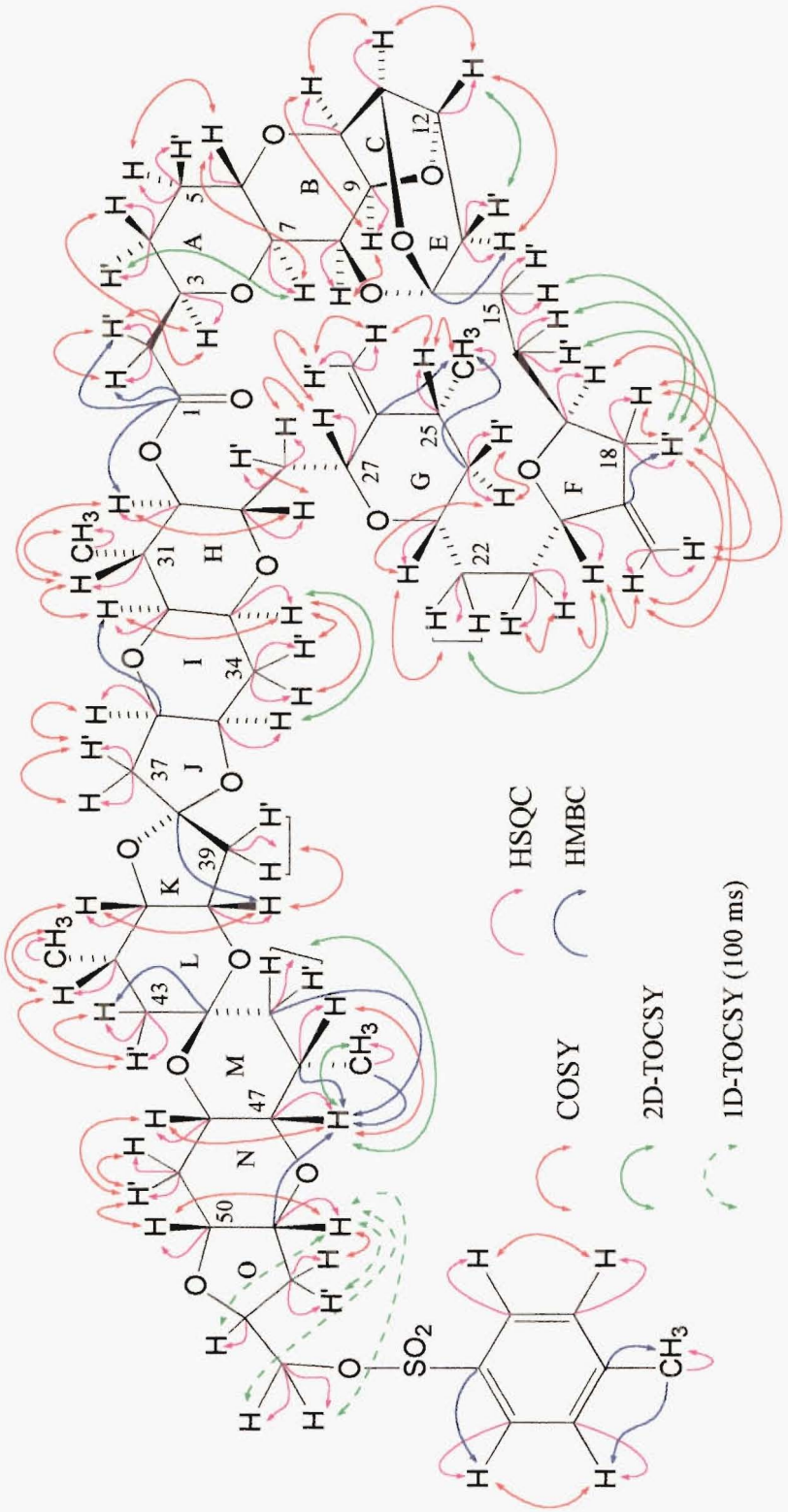
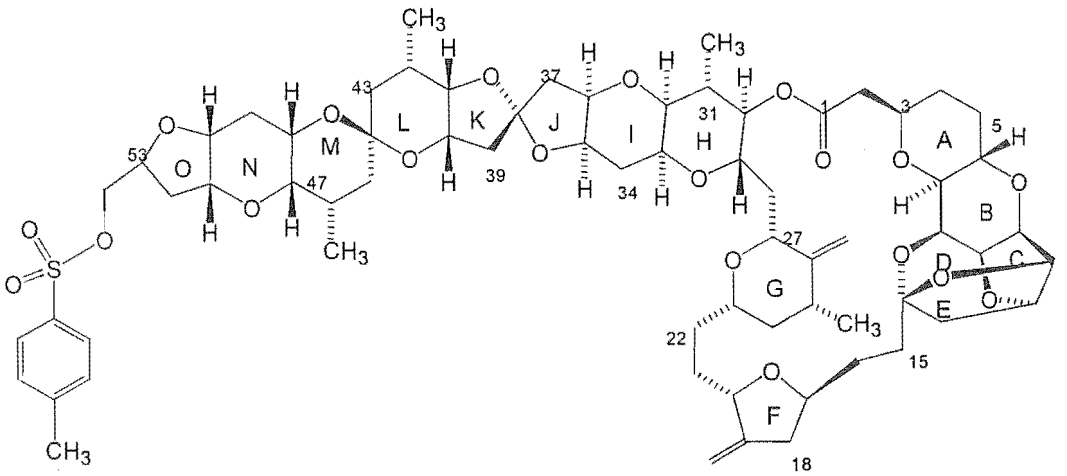


Figure 6.3.2 APT Spectrum of Norhomohalichondrin B Tosylate (**6.8**)

Figure 6.3.3 Norhomohalichondrin B Tosylate – Important NMR Correlations



These data confirmed the connectivities and chemical shifts of the A-L rings were identical to those of norhomohalichondrin B (**4.17**). TOCSY correlations between the H47 resonance (δ_H 3.02) and δ_H 1.37, 2.13, 0.86, 3.48 and 1.74 allowed the assignment of H45/45', H46, CH_3-46, H48 and H49. A COSY correlation between the H49 resonance and δ_H 2.08 allowed the assignment of the geminal H49' resonance. The H50 resonance (δ_H 3.81) was likewise assigned from a COSY correlation to the H49 resonance. A COSY correlation between H50 and a proton resonating at δ_H 3.96 allowed the assignment of H51. A 1D TOCSY with a mixing time of 100 ms was used to detect the resonances of H52 (δ_H 1.87), H52' (δ_H 2.02), H53 (δ_H 4.32), H54 (δ_H 4.02) and H54' (δ_H 4.08). The molecular formula of $C_{67}H_{90}O_{20}S$ determined by HRFABMS was consistent with formation of the tosylate **6.8**.



norhomohalichondrin B tosylate (**6.8**)

6.3.2.7 Formation of Norhomohalichondrin B Azide (**6.9**)

Norhomohalichondrin B tosylate (**6.8**, 8.5 mg) was stirred with an excess of NaN_3 in DMF at 40°C for 19 hours. The EtOAc extract was analysed by reverse phase (C18) HPLC (mobile phase 70% CH_3CN/H_2O) with RI detection. Seventy per cent conversion of tosylate to azide was observed. The reaction with sodium azide was repeated, and progress monitored by HPLC. When the azide peak in the RI chromatogram had maximised, and only trace levels of tosylate remained, the reaction was diluted with

water and extracted into EtOAc. The extract was washed, dried and the solvent evaporated to yield norhomohalichondrin B azide (**6.9**, 5.4 mg).

The most notable difference in the ^{1}H NMR spectrum of **6.9** (Figure 6.3.4) compared to that of the tosylate **6.8** was a multiplet at δ_H 3.12, which overlaps with the H32 and H47 resonances. Differences in the regions of δ_H 3.60-4.00 and δ_H 4.25-4.50 were also apparent.

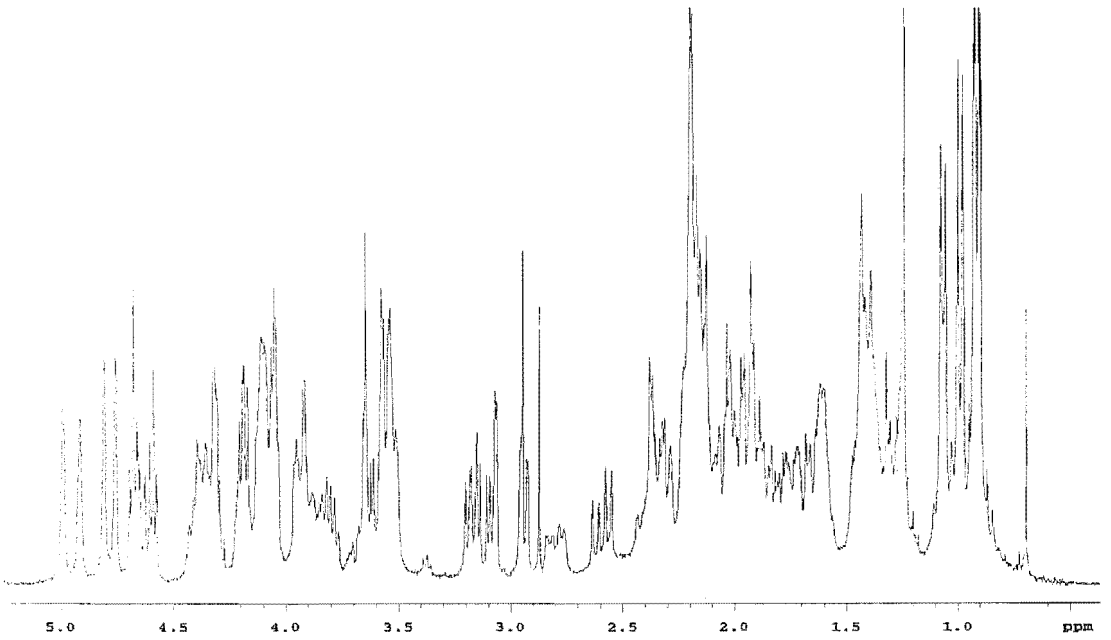


Figure 6.3.4 ^{1}H NMR Spectrum of Norhomohalichondrin B Azide (**6.9**)

An array of NMR experiments were performed on **6.9** *viz* COSY, 1D-TOCSY, 2D-TOCSY (mixing time 100 ms), HSQC, HMBC and APT (Figure 6.3.5) experiments. The ^{1}H and ^{13}C NMR data extracted from these experiments are listed in Table 6.3.3 and Table 6.3.4. The important NMR correlations are shown in Figure 6.3.6.

Table 6.3.3 ^{1}H NMR Data for Norhomohalichondrin B Azide (6.9)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H20**	4.37	**H37**	1.89
H2'	2.59	**H21**	1.38	**H37'**	2.33
H3	3.88	**H21'**	1.87	**H39**	2.18
H4	1.35	**H22**	1.60	**H39'**	2.18
H4'	1.73	**H22'**	1.60	**H40**	3.92
H5	1.35	**H23**	3.53	**H41**	3.58
H5'	2.08	**H24**	1.03	**H42**	2.37
H6	4.33	**H24'**	1.68	**CH_3-42**	0.92
H7	2.94	**H25**	2.20	**H43**	1.29
H8	4.30	**CH_3-25**	1.07	**H43'**	1.42
H9	4.05	**26=CH_2**	4.76	**H45**	1.41
H10	4.18	**26'=CH_2**	4.81	**H45'**	1.41
H11	4.59	**H27**	3.51	**H46**	2.18
H12	4.68	**H28**	1.90	**CH_3-46**	0.91
H13	1.94	**H28'**	2.00	**H47**	3.07
H13'	2.14	**H29**	4.18	**H48**	3.54
H15	1.59	**H30**	4.66	**H49**	1.80
H15'	2.16	**H31**	2.02	**H49'**	2.17
H16	1.42	**CH_3-31**	0.99	**H50**	3.95
H16'	2.16	**H32**	3.17	**H51**	4.05
H17	4.09	**H33**	3.80	**H52**	1.97
H18	2.25	**H34**	1.78	**H52'**	1.97
H18'	2.80	**H34'**	2.15	**H53**	4.40
19=CH_2	4.92	**H35**	4.11	**H54**	3.12
19'=CH_2	4.99	**H36**	4.11	**H54'**	3.59

[a] The symbol ' represents the less shielded proton of a geminal pair.

[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 6.3.4 ^{13}C NMR Data for Norhomohalichondrin B Azide (**6.9**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.0	C20	75.1	C37	43.5
C2	40.5	C21	29.5	C38	112.3
C3	73.7	C22	32.1	C39	42.6
C4	30.8	C23	74.8	C40	70.7
C5	30.1	C24	43.4	C41	79.4
C6	68.2	$\underline{C}$-CH_3-25	36.0	$\underline{C}$-CH_3-42	25.7
C7	77.7	C-$\underline{C}H_3$-25	18.1	C-$\underline{C}H_3$-42	17.7
C8	74.3	26$\underline{C}$=CH_2	151.6	C43	37.1
C9	73.9	26C=$\underline{C}H_2$	104.1	C44	96.6
C10	76.3	C27	73.5	C45	37.1
C11	82.1	C28	37.0	$\underline{C}$-CH_3-46	28.9
C12	81.1	C29	71.2	C-$\underline{C}H_3$-46	17.2
C13	48.4	C30	76.7	C47	73.1
C14	110.0	$\underline{C}$-CH_3-31	36.6	C48	63.6
C15	34.5	C-$\underline{C}H_3$-31	15.1	C49	31.4
C16	28.2	C32	77.6	C50	74.3
C17		C33	66.6	C51	76.9
C18	38.8	C34	29.1	C52	37.4
19$\underline{C}$=CH_2	151.4	C35		C53	76.5
19C=$\underline{C}H_2$	104.4	C36		C54	53.9

[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

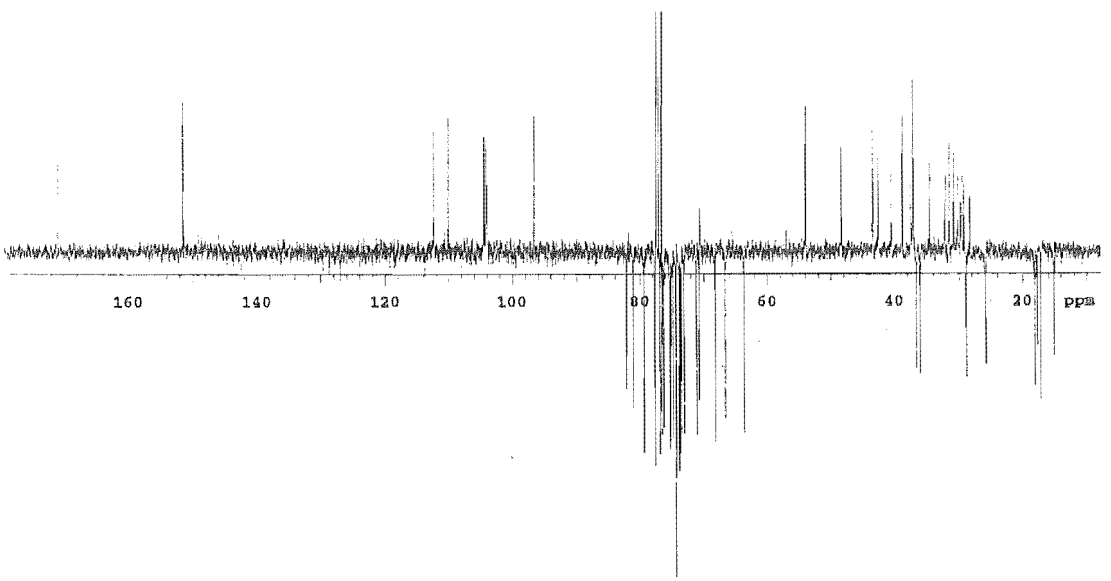
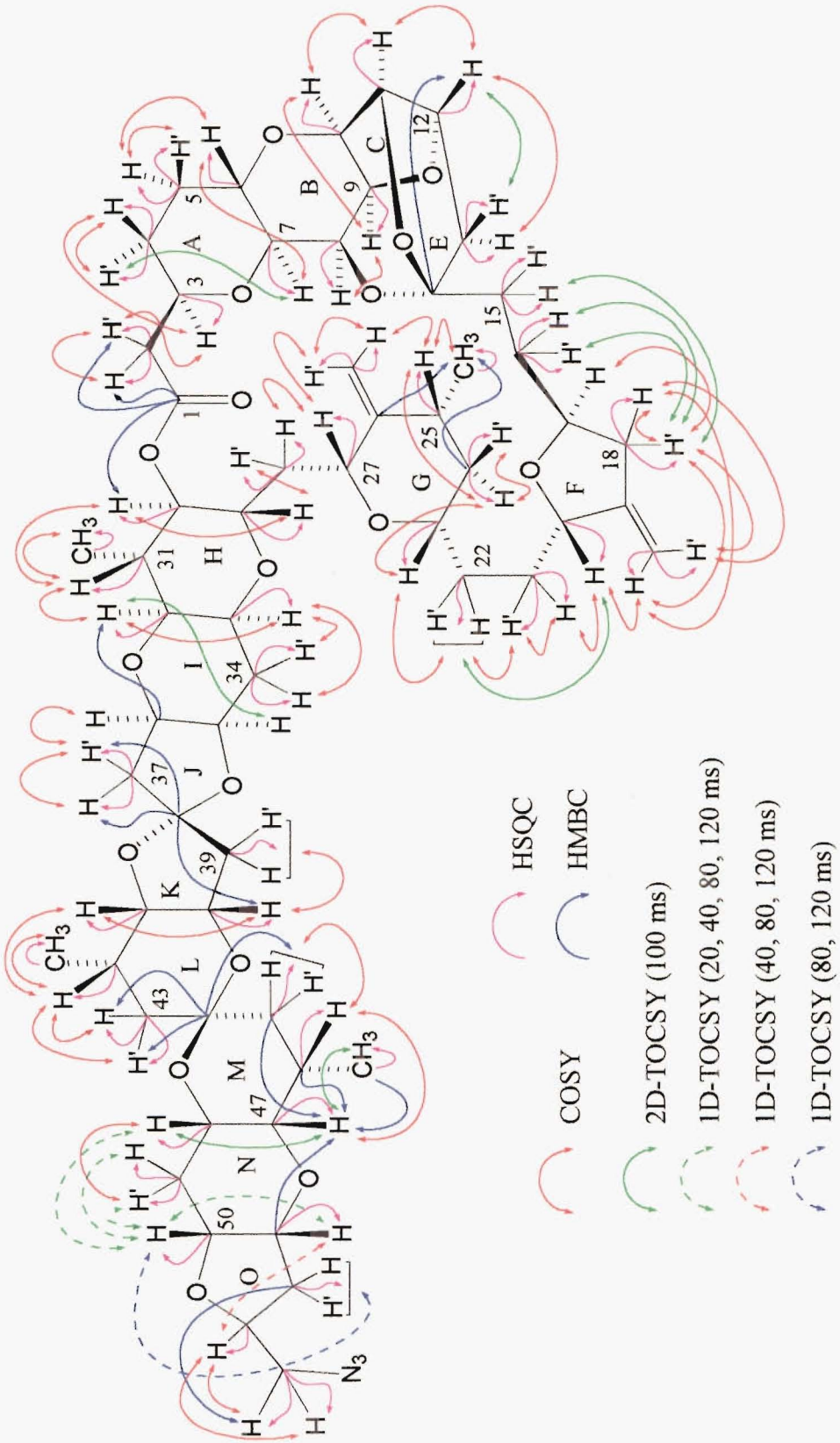


Figure 6.3.5 APT Spectrum of Norhomohalichondrin B Azide (**6.9**)

Figure 6.3.6 Norhomohalichondrin B Azide – Important NMR Correlations



These data confirmed that the connectivities and chemical shifts of the A-M rings were identical to those of norhomohalichondrin B (**4.17**). The new multiplet at δ_H 3.12 was assigned to the H54 proton. Three selective 1D-TOCSY experiments with variable mixing times (10, 20, 40, 80 and 120 ms) were run on **6.9** to confirm this assignment, and enable the assignment of the chemical shift and connectivity of the O-ring and terminal proton resonances. A 1D-TOCSY experiment for the selective irradiation of the H54 proton is shown in Figure 6.3.7. Correlations to the H53 (δ_H 4.40) and geminal H54' (δ_H 3.59) proton resonances were clearly observed with a mixing time of 20 ms. The H51 and H52/52' resonances were observed as the mixing time was increased to 80 ms.

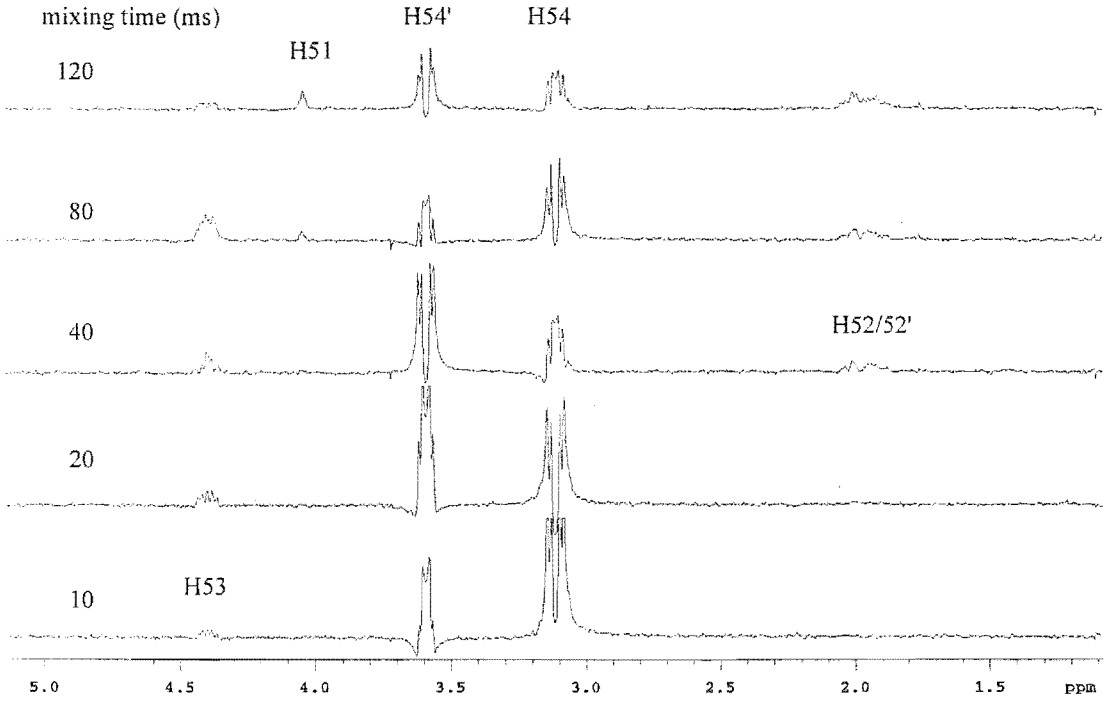


Figure 6.3.7 1D-TOCSY Spectra for Selective Irradiation of H54 with Variable Mixing Times

Irradiation of H53 in a 1D-TOCSY experiment (Figure 6.3.8) also perturbed the H20 proton. It was therefore important to have the spin-system involving H20 well characterised to provide certainty to assignments arising from the irradiation of H53 and H20. Correlations to the H54 and H54' protons were observed with a mixing time of

just 10 ms. Correlations to the H52/52', H51 and finally H50 (mixing time 120 ms) protons were seen as the mixing time was increased. The assignment of these protons was confirmed by a 1D-TOCSY experiment selectively irradiating H50. Correlations to H51, H52/52', H49, H49' and H48 were observed. The molecular formula of $C_{60}H_{83}O_{17}N_3$ determined by HRFABMS confirmed formation of the azide **6.9**.

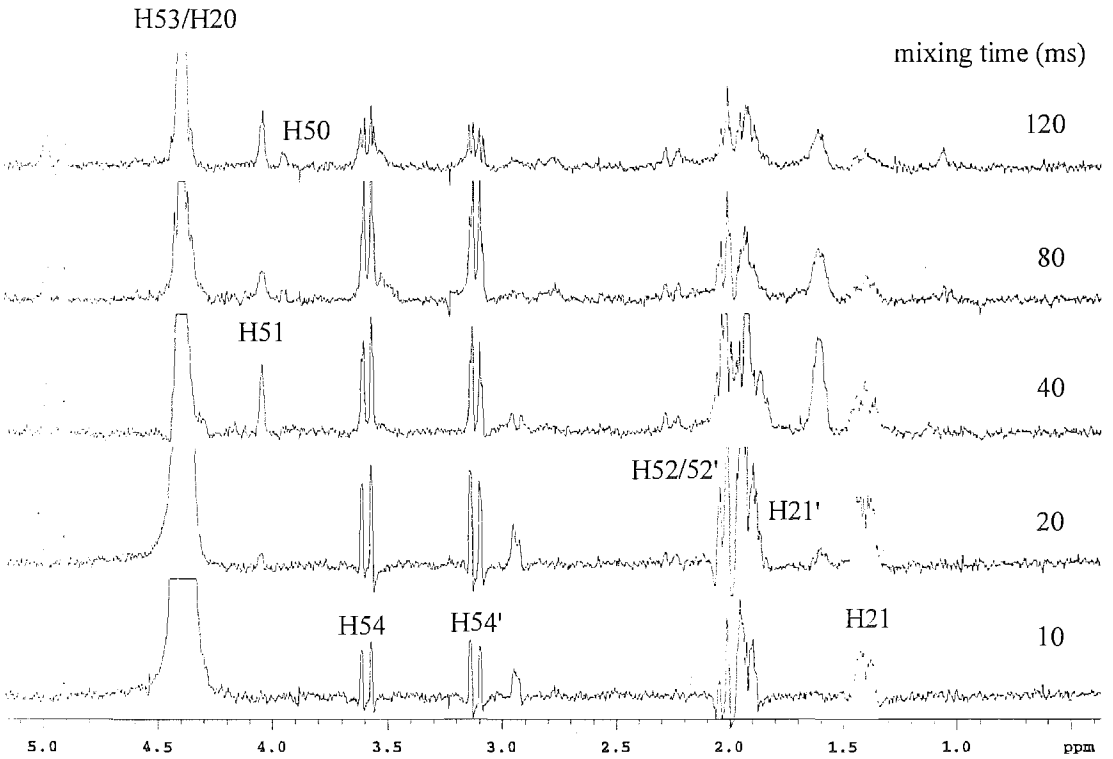
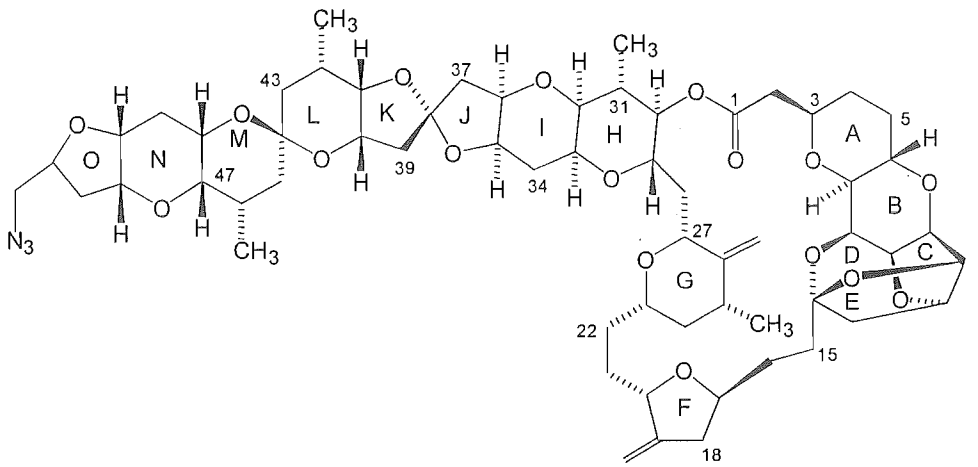


Figure 6.3.8 1D-TOCSY Spectra for Selective Irradiation of H53 with Variable Mixing Times



norhomohalichondrin B azide (**6.9**)

6.3.2.8 Reduction of Norhomohalichondrin B Azide (**6.9**)

Hydrogenation of norhomohalichondrin B azide (**6.9**, 2.0 mg) was carried out with Lindlar catalyst in ethanol at room temperature and atmospheric pressure. The reaction was monitored by reverse phase (C18) HPLC using a mobile phase of 70% CH_3CN/H_2O. After 32 hours the reaction was filtered through Celite and the solvent evaporated to give an orange oil. The H54 resonance was significantly reduced in the ^1H NMR spectrum of the product. The exocyclic methylene resonances were present, indicating that the olefinic groups remained intact. Resonances associated with an unidentified component were observed in the region of δ_H 0.75-0.85. Reverse phase (C18) chromatography in a Pasteur pipette was used to separate the components. The column was eluted with a gradient from 50% CH_3CN/H_2O to CH_3CN and then stripped with CH_2Cl_2. The orange material remained on the C18 column. Trace levels (< 0.1 mg) of the azide **6.9** were recovered. No product was obtained, even after stripping the column with MeOH/TFA.

Hydrogenation of the remaining azide (1.4 mg) was carried out using Lindlar catalyst in ethanol at room temperature and atmospheric pressure for 41 hours. The reaction was filtered through Celite and the solvent evaporated to yield an orange oil. The disappearance of the H54 resonance from the ^1H NMR spectrum (Figure 6.3.9) of the product indicated that reaction of the azide had occurred. The extra resonances upfield of δ_H 0.90 were present once again.

Owing to the difficulties encountered previously in trying to purify the product it was decided to leave the purification step until after reaction of the amine with polymer precursor. It was thought that the large difference in molecular weight of the components would enable purification by gel permeation chromatography on LH20.

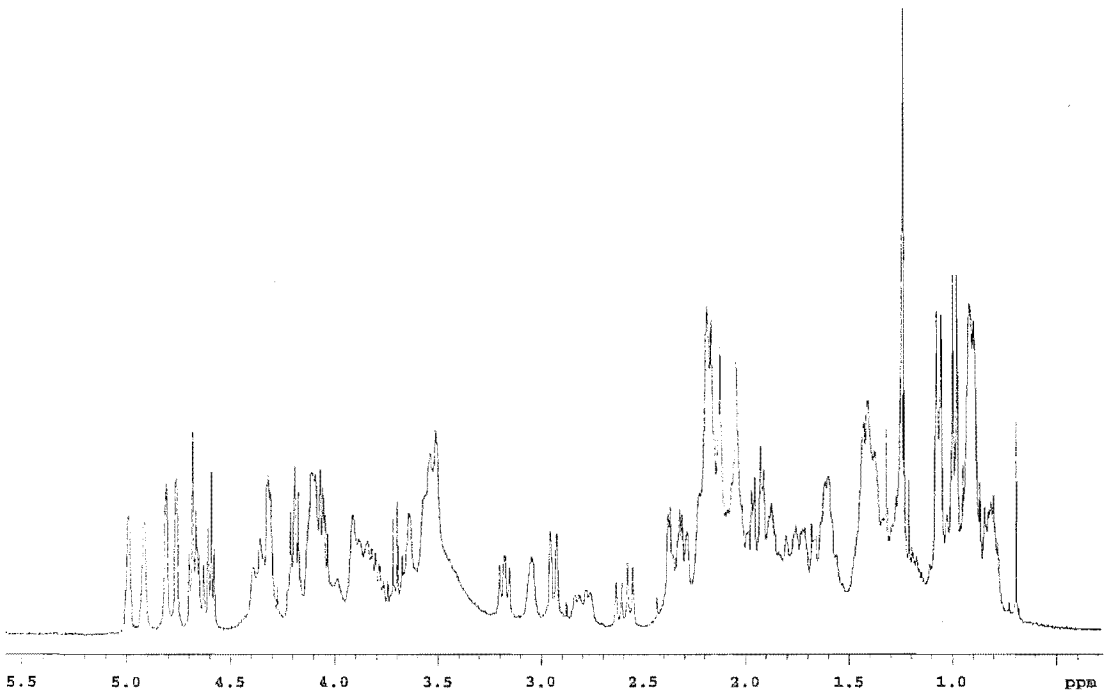


Figure 6.3.9 ^{1}H NMR Spectrum of Norhomohalichondrin B Amine (**6.10**)

COSY, 1D-NOESY, 2D-TOCSY (mixing time 100 ms), HSQC, HMBC and APT experiments were performed on norhomohalichondrin B amine (**6.10**). These data enabled the partial assignment of the ^{1}H NMR and ^{13}C NMR resonances of **6.10** to be achieved. The ^{1}H and ^{13}C spectral data are collated in Table 6.3.5 and Table 6.3.6 respectively. Important 2D NMR correlations are shown in Figure 6.3.10.

Table 6.3.5 ^{1}H NMR Data for Norhomohalichondrin B Amine (**6.10**)

Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]	Proton[a]	δ ppm[b]
H2	2.34	**H20**	4.37	**H37**	1.89
H2'	2.59	**H21**	1.39	**H37'**	2.79
H3	3.88	**H21'**		**H39**	2.18
H4	1.35	**H22**	1.60	**H39'**	2.18
H4'	1.72	**H22'**	1.60	**H40**	3.92
H5	1.37	**H23**	3.54	**H41**	3.56
H5'	2.09	**H24**	1.03	**H42**	
H6	4.34	**H24'**	1.67	**CH_3-42**	0.90
H7	2.94	**H25**	2.20	**H43**	1.40
H8	4.32	**CH_3-25**	1.07	**H43'**	
H9	4.06	**26=CH_2**	4.76	**H45**	1.40
H10	4.20	**26'=CH_2**	4.81	**H45'**	
H11	4.60	**H27**	3.52	**H46**	2.17
H12	4.68	**H28**	1.94	**CH_3-46**	0.89
H13	1.98	**H28'**		**H47**	3.04
H13'	2.16	**H29**	4.19	**H48**	3.51
H15		**H30**	4.66	**H49**	1.80
H15'		**H31**	2.01	**H49'**	2.15
H16		**CH_3-31**	0.99	**H50**	
H16'		**H32**	3.18	**H51**	4.01
H17	4.09	**H33**	3.80	**H52**	
H18	2.25	**H34**	1.77	**H52'**	
H18'	2.80	**H34'**	2.15	**H53**	
19=CH_2	4.92	**H35**	4.11	**H54**	
19'=CH_2	4.99	**H36**	4.11	**H54'**	

[a] The symbol ' represents the less shielded proton of a geminal pair.

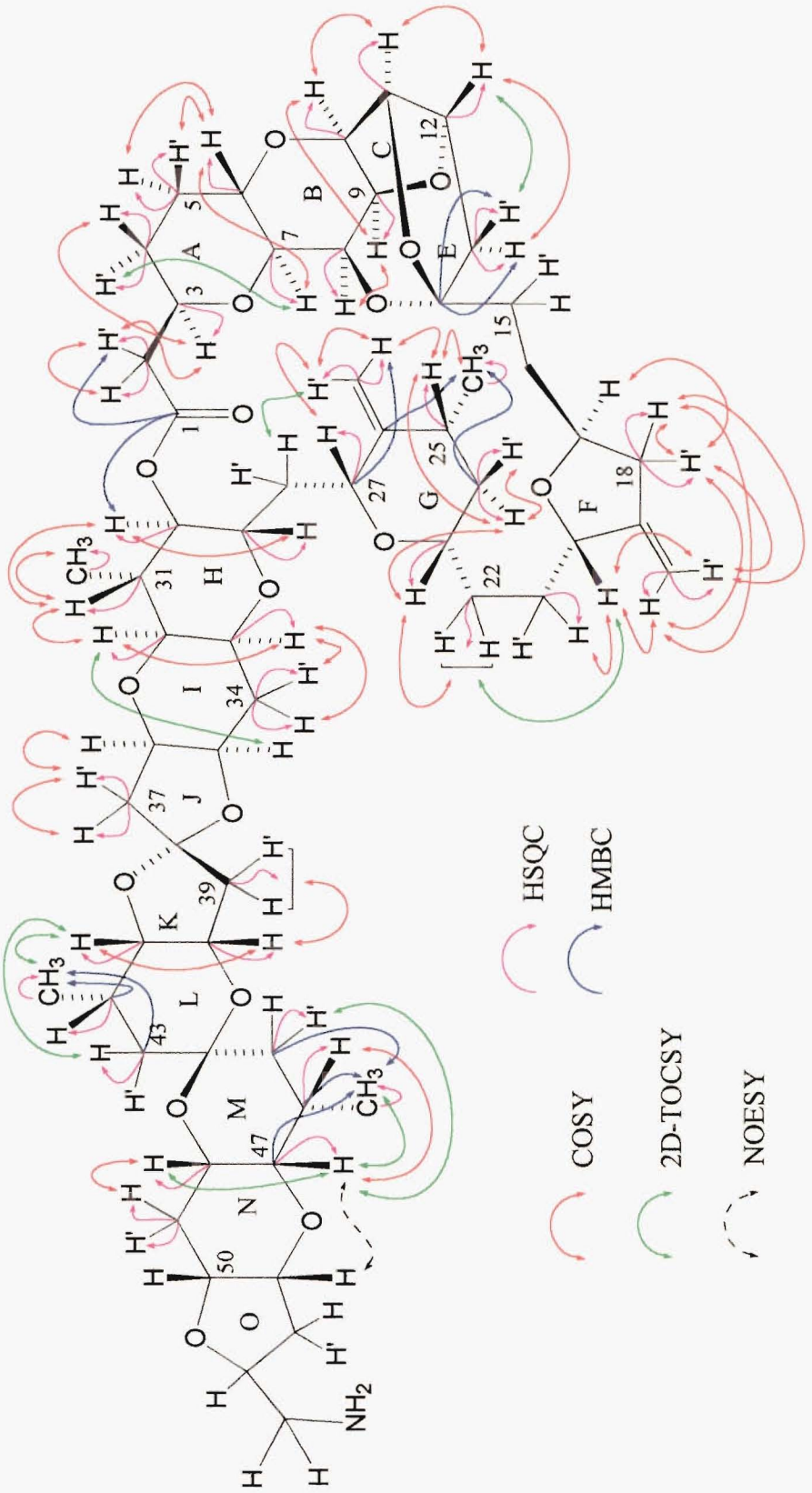
[b] Data recorded at 23°C in $CDCl_3$ at 300 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_H 7.26.

Table 6.3.6 ^{13}C NMR Data for Norhomohalichondrin B Amine (**6.10**)

Carbon	δ ppm[a]	Carbon	δ ppm[a]	Carbon	δ ppm[a]
C1	171.0	C20		C37	43.5
C2	40.5	C21	29.5	C38	112.3
C3	73.7	C22	32.1	C39	42.6
C4	30.1	C23	74.4	C40	70.7
C5	29.8	C24	43.5	C41	79.4
C6	68.3	C-CH_3-25	36.0	C-CH_3-42	25.8
C7	77.6	C-CH_3-25	18.1	C-CH_3-42	17.8
C8	74.4	26C=CH_2	151.6	C43	
C9	73.9	26C=CH_2	104.1	C44	96.7
C10	76.3	C27	73.5	C45	
C11	82.1	C28		C-CH_3-46	28.8
C12	81.1	C29	71.2	C-CH_3-46	17.2
C13	48.4	C30	77.0	C47	73.2
C14	110.0	C-CH_3-31	36.6	C48	63.6
C15	34.5	C-CH_3-31	15.1	C49	30.8
C16	28.3	C32	77.7	C50	
C17		C33	66.6	C51	
C18	38.8	C34	29.1	C52	
19C=CH_2	151.4	C35		C53	
19C=CH_2	104.4	C36		C54	

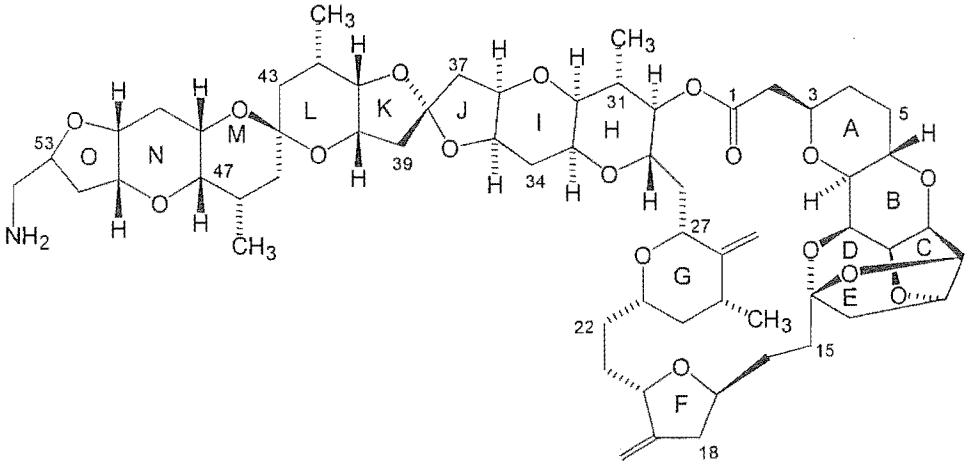
[a] Data recorded at 23°C in $CDCl_3$ at 75 MHz with chemical shifts in ppm and referenced to $CHCl_3$, δ_C 77.0.

Figure 6.3.10 Norhomohalichondrin B Amine - Important NMR Correlations



These data confirmed the connectivity and chemical shifts of the A-M rings were comparable to those of the azide **6.9**. Unfortunately the complex overlapping nature of the COSY and 2D-TOCSY spectra of the amine **6.10** made the assignment of H54/54' and the majority of the O-ring protons impossible. The H51 proton was assigned from the observation of an NOE enhancement arising from selective irradiation of the H47 proton in a 1D-NOESY experiment.

The molecular formula of $C_{60}H_{85}O_{17}N$ determined by HRFABMS confirmed that reduction of the azide had occurred.



norhomohalichondrin B amine (**6.10**)

6.4 Polymer Therapeutics

6.4.1 Introduction

Before the attachment of norhomohalichondrin B amine (**6.10**) to the polymer precursor could be undertaken, a method of quantifying the amount of amine attached had to be established. The lack of a UV chromophore prevented direct spectroscopic measurement of the attached amine. The measurement of displaced *p*-nitrophenoxide (ONp) offered the only means of determining the quantity of amine bound to the polymer. The small scale of the reaction also meant that it was not possible to purify

the polymer product by the usual method of crystallisation. The considerable difference in size between the polymer, the halichondrin amine and the free ONp was exploited by using size exclusion chromatography to separate the components.

6.4.2 Determination of ε_{400} for *p*-Nitrophenoxide

The absorption of ONp at 400 nm was measured in duplicate at six different concentrations. The two absorbance readings above 2.0 were excluded as these lay outside the range at which measurements were going to be made. The molar absorbtivity coefficient (ε) for *p*-nitrophenoxide (ONp) at 400 nm was determined using Beer's Law as 13097 $M^{-1}cm^{-1}$.

6.4.3 Trial Reaction

A series of reactions were undertaken to optimise conditions for attachment of an amine to the polymer precursor. The effect of solvent (DMSO) and triethylamine (TEA) on absorption was investigated. The absorption of ONp at 400 nm was found to be pH dependent, while the addition of DMSO had no effect. In order to minimise the variation in absorbance with variation in TEA, phosphate buffered saline (pH 7.68) was used as the diluent for spectroscopic measurements. Absorbance measurements were made immediately upon diluting the reaction mixtures with buffer to minimise hydrolysis of unreacted ONp. The amount of free ONp contaminant in the polymer precursor was also determined, so a correction could be made when calculating the quantity of ONp released.

The polymer precursor in DMSO was treated with an excess of 1-amino-2-propanol for 10 minutes. The absorbance of an aliquot of the reaction at 400 nm was measured. From this, the ONp content of the polymer precursor was calculated as 2.7 mol%. This is lower than the 3.98 mol% determined previously.[114] However the value is similar,

and reaction of amine with polymer precursor for a longer period of time should ensure release of all the ONp.

The polymer precursor (5.0 mg) was stirred with 1-amino-2-propanol and TEA in DMSO for 23 hours at room temperature. An aliquot of the reaction solution was diluted to 1 mL with phosphate buffered saline. The absorbance of this dilute solution at 400 nm was 1.1938. This value was used to calculate a side chain content of 4.5 mol% (see Appendix 1 for calculations).

The remaining reaction mixture was diluted to 1 mL with MeOH and purified on an LH20 column using MeOH as the eluent. The column was plumbed into a single wavelength spectrometer, and the elution of the polymer and free ONp at 206 nm observed as separate, distinct peaks. This exercise illustrated that the polymer could be resolved from the free ONp on LH20. Reduction of the flowrate to *ca* 0.5 mL/minute would enhance the separation of these two components.

6.4.4 Attachment of Norhomohalichondrin B Amine to Polymer Precursor

Polymer precursor (5.0 mg), norhomohalichondrin B amine (**6.10**, 1.4 mg) and one equivalent of TEA were stirred for 23 hours in DMSO. A small aliquot (6 μL) of the reaction solution was diluted to 1 mL with phosphate buffered saline. The absorbance of this dilute solution at 400 nm was 0.6733. This equates to the addition of *ca* 0.8 mg of norhomohalichondrin B amine (**6.10**) to the polymer (2.2 mol%, 13.7 weight%).

An excess of 1-amino-2-propanol was added to the reaction solution. Stirring was continued for a further 16 hours before another aliquot (6 μL) was removed, diluted and the absorbance read as described above. The absorbance of the diluted reaction mixture at 400 nm was now 1.2134. From this, a value of 4.6 mol% of side chain was calculated.

The reaction mixture was diluted with MeOH to give a final volume of 1 mL and then applied to an LH20 column. The column was eluted with MeOH at a rate of 0.5 mL/minute. Fractions were collected at 2 minute intervals. Fractions containing polymer (8-14) were identified by their ^{1}H NMR spectra. Dragendorff's spray reagent[125] was used to identify fractions containing free amine (14-16). Fractions containing purely polymer-bound amine were combined and the solvent evaporated to give the product (4.12 mg).

HSQC, COSY and 2D-TOCSY (mixing time 40 ms) NMR experiments were run on the halichondrin polymer conjugate. COSY correlations belonging to the halichondrin part of the polymer conjugate are shown in Table 6.4.1. No halichondrin-type correlations could be seen in the HSQC and 2D-TOCSY spectra.

Table 6.4.1 Observed COSY Correlations for Halichondrin Polymer Conjugate

COSY Correlation (H-H)		
6-7	18'-CH_2=19'	30-31
7-8	20-CH_2=19'	31-CH_3-31
10-11	20-CH_2=19'	31-32
12-13	20-21	36-37'
18-18'	25-CH_3-25	

6.4.5 Biological Activity

The biological activity of norhomohalichondrin B amine (**6.10**) and the halichondrin polymer conjugate were evaluated against the P388 murine leukemia cell line. The polymer conjugate was also submitted at a concentration equivalent to 0.01 mg/mL of amine to compare the activity of the polymer-bound amine with the free amine **6.10**. Norhomohalichondrin B (**4.17**) was assayed to allow a comparison of activity between

the amine derivative **6.10** and the parent alcohol. The assay results are shown in Table 6.4.2.

Table 6.4.2 *In Vitro* Cytotoxicities of Selected Halichondrins

Entry	Compound	P388 IC_{50} (ng/mL)
1	**norhomohalichondrin B (4.17)**	1.9
2	**norhomohalichondrin B amine (6.10)**	1.4
3	**halichondrin polymer conjugate**[a]	6.1
4	**halichondrin polymer conjugate**	46.3
5	**aminopropanol polymer conjugate**	4265
6	**norhomohalichondrin B tosylate (6.8)**	1.8

[a] submitted at concentration of norhomohalichondrin B amine in the polymer

The first three results in Table 6.4.2 are the average of seven independent assay results for each of those samples. The amine **6.10** and tosylate **6.8** derivatives (entries 2 and 6) were as active as the parent norhomohalichondrin B (**4.17**). This result is consistent with previous observations that alteration of the terminus of the halichondrin skeleton does not have significant effect on activity.

Taking into account the inherent variability of the biological assay, the activity of the polymer bound amine against the P388 cell line was comparable to that of norhomohalichondrin B amine (**6.10**) when assayed at equivalent amine concentrations (entries 2 and 3). The activity of the halichondrin polymer conjugate assayed by total weight (entry 4) is an order of magnitude less active than the free amine **6.10**. This result gives a good indication as to the purity of the halichondrin polymer. In order to achieve a comparable result should the halichondrin polymer be contaminated with free amine, a large proportion of the sample would need to be free amine. The activity of the halichondrin polymer is encouraging evidence that the norhomohalichondrin amine is

being released from the polymer. A more extensive *in* vitro and *in vivo* evaluation of the halichondrin polymer is in progress.

6.5 Summary

A route to a halichondrin amine has been established. This amine (**6.10**) was subsequently incorporated into a polymer drug conjugate. Preliminary biological testing against the *in vitro* P388 cell line indicates that the polymer drug conjugate has a level of cytotoxicity equivalent to that of the unbound norhomohalichondrin B amine (**6.10**). The halichondrin-containing polymer drug conjugate represents an exciting advance in the future prospects of the halichondrins as anticancer therapeutics.

Chapter 7

Experimental

7.1 General Methods

7.1.1 Nuclear Magnetic Resonance (NMR)

Proton detected NMR spectra were recorded on a Varian Unity 300 spectrometer at 23 °C, operating at 300 MHz. Carbon detected NMR spectra were recorded on a Varian XL300 spectrometer at 23 °C, operating at 75 MHz. Other NMR experiments described in this thesis *viz* 1D and 2D-TOCSY, NOE, COSY, NOESY and the reverse detected HSMQC, HSQC and HMBC experiments were recorded on the Unity spectrometer, at 300 MHz. For the work detailed in Chapters 2, 4 (except Section 4.7) and 5 the instrument was fitted with a Nalorac Z.spec MID300 3 mm Indirect Detection Probe. For the work detailed in Section 4.7 and Chapter 6 the instrument was fitted with a Pulsed Field Gradient MLD driver with a 5 mm Indirect Detection Probe. Chemical shifts in this thesis are described in parts per million (ppm), on the δ scale, and were referenced to the appropriate solvent peaks: $CDCl_3$ referenced to $CHCl_3$ at δ_H 7.26 (^1H) and $CHCl_3$ at δ_C 77.0 (^{13}C). All spectra, unless stated, were obtained in $CDCl_3$ with 0.1% C_5D_5N to remove any traces of acid from the solvent. ^1H NMR spectra were recorded using an acquisition time (AT) of 2.0 s; ^{13}C NMR spectra were recorded using an AT of 0.878 s. APT experiments were recorded using an AT of 0.878 s, D1=0 s, D2=0.07 s and D3=0.01 s. All difference NOE experiments were recorded in undegassed solutions, with an AT of 1.0 s and an irradiation time (D2) of 2.0 s. The decoupler was offset 10 000 Hz for the control experiments. NOESY experiments were run using an AT of 0.384 s and a mixing time of 0.30 s. COSY experiments were

recorded using an AT of typically 0.2 s and a relaxation delay (D1) of 1.0 s. 2D-TOCSY spectra were recorded using an AT of typically 0.2 s and a relaxation delay (D1) of 1.0 s, with mixing times indicated in the discussion. 1D TOCSY were recorded using a AT of 2.0s, with or without a D1 of 1.0 s. HSMQC experiments were recorded with an AT of 0.178 s, a D1 of 0.333 s and J_{CH} of 145 Hz. HSQC experiments with the Pulsed Field Gradient system were run with an AT of typically 0.14 s, a D1 of 1.0 s and $J_{C\text{-}H}$ of 140 Hz. HMBC experiments were recorded with an AT of typically 0.18 s, a relaxation delay of 0.333 s, J=130 Hz and a $^{n}J_{CH}$ of 8.3 Hz. HMBC experiments with the Pulsed Field Gradient system were run with an AT of *ca* 0.2 s, a D1 of 1.0 s, a J of 140 Hz and a $^{n}J_{CH}$ of 8.3 Hz.

7.1.2 Mass Spectrometry

Mass spectrometry was performed on a Kratos MS80 RFA Mass Spectrometer operated at 4000 V. All halichondrin derivatives were analysed by the soft ionisation technique of fast atom bombardment (FAB). FAB was performed with an Ion Tech ZNIIFN ion gun using Xe as the reagent gas, operating at 8 kV and 2 mA with an *m*-nitrobenzyl alcohol (NOBA) matrix. Electron impact ionisation at 70 eV was used to analyse some of the trial reaction products. Some samples (stated) were sent to Lewis Pannell (NIDDK, NIH) in the USA for HRFABMS analysis. Where the matrix varied from NOBA this will be stated.

7.1.3 High Performance Liquid Chromatography

The HPLC work described in this thesis was performed on either one of three instruments. A Philips PU4100 Liquid Chromatograph equipped with a Philips PU4120 Diode Array Detector interfaced to a PC General 486 computer running Philips PU6003 Diode Array Detector System Software (V3.0), and a Philips PPG 3160/10 colour plotter was used for analytical and small scale preparative HPLC described in Chapters 2-5. Solvents were degassed using a flow of helium. The remaining analytical HPLC

(Chapter 6) was performed on a Shimadzu VP system. The complete setup involved a Shimadzu LC-10AC VP liquid chromatograph coupled to a SIL-10A VP autoinjector, a CTO-10A VP column oven set to 40 °C, a SPD-M10A VP diode array detector and a RID-10A VP Refractive index detector. This system was controlled by Shimadzu CLASS-VP (Version 5.02) software. A Shimadzu degasser was utilised for the degassing of all solvents used in this machine. Both instruments were equipped with a reverse-phase Brownlee Labs C18 column (dimensions 220 mm (L) × 4.6 mm (ID), 5 μm particle size). Stated pre-mixed solvent mixtures of acetonitrile (BDH HiperSolv™ 'Far UV' grade) and water (purified using a MilliQ deionising system) were used with a solvent flow rate of 1 mL/minute. A Shimadzu LC-4A instrument equipped with a Shimadzu UV Spectrotometric Detector SPD-2AS (wavelength λ=199 nm) and Hewlett Packard 3390A integrator was used for preparative reverse phase work using a Rainin Dynamax-60A C18 column (dimensions 250 mm (L) × 21.4 mm (ID), 8 μm average particle size (APD)), the stated acetonitrile (CH_3CN) and water mixture with a flow rate of 10 mL/minute.

7.1.4 Column Chromatography

All column chromatography was performed with glass columns of stated dimensions. Solvents used were all of commercial grade, distilled once in glass distillation apparatus, except MeOH, which was distilled twice. "Flash" columns were run under N_2 gas (oxygen free) pressure (0.5 kPa).

Silica flash chromatography was performed on Merck silica gel 60 (230-400 mesh).[126] Normal phase column chromatography of halichondrin material was performed using Merck DIOL (40 μm APD). Small scale column chromatography was carried out using disposable Adsorbex 200 mg Merck DIOL cartridges. Sephadex LH20 (Pharmacia Biotech AB) presoaked overnight in CH_2Cl_2 was used for gel permeation chromatography.

Octadecyl (C18) reverse phase packing used for preparative and flash column chromatography was either Bakerbond (40 μm) or prepared from silica gel (Davisil, 35-70 μm) using a solution of octadecyltrichlorosilane (2% *v/v*, EGA-Chemie) in carbon tetrachloride.[127] The silica gel was stirred gently overnight with a teflon coated magnetic follower before washing with carbon tetrachloride (×2). Unreacted chloro groups were substituted by washing with dry MeOH. A carbon tetrachloride solution of trimethylchlorosilane (2% *v/v*, Aldrich) was used to end-cap any unreacted hydroxy groups. The reverse phase material was finally washed with CH_2Cl_2 (×2) and MeOH (×2). Small scale reverse phase (C18) column chromatography was performed using either 500 mg Bakerbond spe or 200 mg BondElut disposable C18 cartridges.

7.1.5 Thin Layer Chromatography

The analytical silica TLC described in this thesis was performed using Merck silica gel 60 F_{254} aluminium-backed sheets, the silica 0.2 mm in thickness. DIOL analytical TLC was performed using Merck F_{254} glass-backed plates, the DIOL 0.2 mm in thickness. DIOL plates were eluted with the stated MeOH/CH_2Cl_2 mixtures. Silica plates were eluted with PE/EtOAc mixtures as stated. Merck RP-18 F_{254} TLC plates of 0.2 mm were used for the analytical C18 TLC performed in this thesis. The C18 plates were eluted with the stated CH_3CN/H_2O mixtures. TLC plates containing halichondrins were visualised using a phosphomolybdic acid (PMA) in ethanol (EtOH) spray (10% PMA in EtOH, *w/v*), followed by heating at *ca* 80°C for 5 minute to yield characteristic brown spots. Some TLC plates (as stated) were initially visualised under a short-wave (λ 254 nm) lamp before dipping in a potassium permanganate in ethanol/H_2O dip (1:1, 10% *w/v*).

7.1.6 Solvents

Technical grade solvents were distilled prior to use. Methanol was distilled twice. Acetic anhydride was distilled immediately prior to use. 'Hexanes' were prepared from

petroleum ether (10 L, bp 60-70 °C), nitrating with a mixture of conc. HNO_3 (1L) and conc. H_2SO_4 (1L) for two days. After this time the nitrating mixture was removed and the petroleum ether was washed with water (20 L), and dried over $CaCl_2$ overnight. The resulting solution was filtered through alumina and distilled. Dry solvents were obtained using standard methods. Pyridine was refluxed over CaH_2 before distillation. Methanol was distilled from magnesium metal and iodine and stored over activated molecular sieves (4 Å). Tetrahydrofuran was refluxed over sodium metal and benzophenone before distillation. Dichloromethane was refluxed over calcium hydride before distillation. DMSO was dried over molecular sieves overnight. DMF was dried by treating twice overnight with 4 Å molecular sieves, followed by storage over 4 Å sieves. IPA was refluxed over calcium hydride for 3 hours before distillation.

7.2 Work Described in Chapter Two

7.2.1 Introduction

Batch 32, fractions 1-2 (1.19 g) and Batch 33, fractions 1-3 (0.89 g) were **1.28**-containing fractions provided by the Hali-JV project. Batch 32 was processed in four portions through Sephadex LH20 gel (100 g, 1260 mm × 18 mm) presoaked in CH_2Cl_2 overnight. The column was eluted with CH_2Cl_2. An initial fraction (75 mL) was collected followed by a further 10 fractions (10 mL) before the gel was stripped with 1:1 $MeOH/CH_2Cl_2$.

Analytical reverse phase (C18) HPLC (eluent 90% CH_3CN/H_2O) was utilised to identify fractions from LH20 that contained **1.28** and **1.32**. These combined fractions (REL3 25.6-8, 167.2 mg; REL3 27.6-8, 181.6 mg; REL3 28.4-6, 70.5 mg; REL3 29.6-7, 194.2 mg) were divided in 2 and both chromatographed further on LH20 (100 g, 1260 mm × 18 mm). Twenty fractions were collected from each column and analysed by reverse phase (C18) HPLC for **1.28** and **1.32** content. Compound **1.32** was observed in

fractions SH2 101.5 (13.1 mg), SH2 101.6 (31.0 mg), SH2 101.7 (80.4 mg), SH2 104.4 (8.6 mg), SH2 104.5 (24.2 mg) and SH2 104.6 (59.3 mg). A mixture of **1.32** and **1.28** were seen in fractions SH2 101.8 (95.8 mg) and SH2 104.7 (71.0 mg). Compound **1.28** was also present in fractions SH2 101.9 (63.6mg), SH2 101.10 (28.7 mg), SH2 101.11 (4.2 mg), SH2 104.8 (60.2 mg), SH2 104.9 (38.7 mg), SH2 104.10 (9.8 mg) and SH2 104.11 (3.5 mg).

7.2.2 Formation and Isolation

7.2.2.1 HPLC Trial

A mixture of **1.28** and **1.32** (100 μg) in CH_3CN (100μL) was treated with aqueous $HClO_4$ (20 μL; 0.035%) for 35 minutes. Small aliquots (20 μL) were removed and analysed by reverse phase (C18) HPLC (90% CH_3CN/H_2O) after 5, 15, 25 and 35 minutes of reaction. Conversion of **1.32** to **1.28** was complete after 5 minutes. A peak of similar polarity (Rf=500 s) to **1.32** was observed to increase with increased reaction time.

7.2.2.2 ^{1}H NMR Trial

A mixture of **1.28** and **1.32** (1.0 mg) in CH_3CN (80 μL) was treated with aqueous $HClO_4$ (20 μL, 0.035%) for 5 minutes. The reaction mixture was diluted with water and run through a reverse phase (C18) cartridge. The perchloric acid was washed through with H_2O before stripping the halichondrin material off the cartridge with CH_3CN and CH_2Cl_2. A ^{1}H NMR spectrum of the combined organic fractions (REL3 24.2, 1.0 mg) revealed an increase in intensity of the characteristic H55 triplet resonance, 4 clean methyl resonances, and the absence of the characteristic methoxy and H47 resonances of **1.32**.

7.2.2.3 Scaled up Acid Treatment of 53-Methoxyneoisohomohalichondrin B (**1.32**)

The combined fractions SH2 101.5-101.7, 104.5, 104.6 (198.2 mg) in CH_3CN (6 mL) were treated with aqueous $HClO_4$ (1.5 mL, 0.035 %) for 6 minutes. The reaction solution was diluted with H_2O (1.5 mL) and loaded onto a reverse phase (C18) column (10 g). The column was flushed with H_2O (150 mL) to remove the $HClO_4$, and the halichondrin material stripped off the column with CH_3CN (250 mL) and CH_2Cl_2 (100 mL). The combined organic fraction was evaporated *in vacuo* to give a pale yellow oil (SH2 107.1, 174.6 mg). Analysis of the reaction product by reverse phase (C18) HPLC revealed the presence of 2 halichondrin-like species, having end absorption only. The more polar component was the expected **1.28**. The other component eluted at 487 s compared to the retention time for **1.32** of 505 s. The characteristic methoxy resonance of **1.32** was not observed in the ^1H NMR spectrum of the mixture.

7.2.2.4 Acid Treatment of a **1.32/1.28** Mixture

A subsample of SH2 104.7 (5.6 mg) in CH_3CN (250 μL) was treated with aqueous $HClO_4$ (63 μL, 0.035 %) for 1 minute before diluting the reaction mixture with *ca* 400 μL H_2O. The diluted solution was loaded onto a reverse phase (C18) cartridge. The cartridge was washed with H_2O (10 mL) before the halichondrin material was stripped with CH_3CN (8 mL) and CH_2Cl_2 (8 mL). The combined organic fraction was dried *in vacuo* to give isohomohalichondrin B (**1.28**, 6.3 mg).

7.2.2.5 Purification of **2.2**

SH2 107.1 in acetonitrile (50 mg/mL) was separated into its 2 component halichondrins by semipreparative reverse phase (C18) HPLC eluting with 90% CH_3CN/H_2O. A series of 8 *ca* 16 μL injections were made and 2 fractions collected to give SH2 118.1 (**1.28**, 2.2 mg) and SH2 118.2 (**2.2**, 4.0 mg). The identity of the more polar fraction **1.28** was

confirmed by comparison of its ^{1}H NMR spectrum to that of an authentic sample of 1.28.

7.2.3 Structural Elucidation

epi-Isohomohalichondrin B (**2.2**)

^{1}H NMR and ^{13}C NMR data cited in Table 2.3.1 and Table 2.3.2 respectively. HRFABMS MNa^+ 1145.5653 ($C_{61}H_{86}O_{19}Na$ requires 1145.5661).

7.2.4 Acid Equilibria

epi-Isohomohalichondrin B (**2.2**, 3.1 mg) was treated 4:1 $CH_3CN/HClO_4$ (120 μL, 0.035%) for 4h. Small aliquots were removed and analysed by reverse phase (C18) HPLC after 0.25, 0.5, 1, 2, 3 and 4h. An equilibrium of 1:1 **1.28**:**2.2** was reached after 3 hours reaction time.

7.3 Work Described in Chapter Three

7.3.1 Sample Details

95AKLissFeb01 1-18, 95AKLissFeb02 1-18, 95BBLissFeb01 1-37. Samples were combined on the basis of depth for each of the two sites. The resulting five combined samples were Beatrix Bay deep (1591 g), Beatrix Bay mid depth (328 g), Beatrix Bay shallow (483 g), Akaroa deep (500 g), Akaroa shallow (400 g).

7.3.2 Extraction

Beatrix Bay Mid-depth

The thawed sponge was blended with MeOH (300 mL) in a Waring blender. The soupy mixture was stirred overnight. Celite (320 g) wet with 4:1 $MeOH/H_2O$ (660 mL) was

added to the sponge slurry and stirring continued for 30 minutes before filtering. The sponge residue was extracted further with MeOH (4 × 700 mL). H_2O (440 mL) was added to the combined filtrate before partitioning against PE (750 mL + 660 mL). The solvent was evaporated *in vacuo* to afford the aqueous methanol extract.

Akaroa Shallow

The combined sponge was blended in a Waring blender with MeOH (400 mL) and then stirred overnight. Celite (80 g) wet with MeOH (130 mL) and H_2O (30 mL) was added to this slurry and stirred for 30 minutes before filtering. The sponge residue was extracted with MeOH (6 × 200 mL). The MeOH/H_2O ratio of the combined extracts was adjusted to 4:1 with the addition of H_2O (210 mL) before partitioning against PE (380 + 340 mL). The solvent was evaporated *in vacuo* to give the aqueous methanol partition.

Akaroa Deep

The combined sponge samples were blended in a Waring blender with MeOH (400 mL) and stirred overnight. Celite wet with 4:1 MeOH/H_2O (200 mL) was added to the sponge slurry and stirring continued for 30 minutes. The slurry was filtered and the sponge residue extracted with MeOH (6 × 300 mL). H_2O (430 mL) was added to the combined filtrate before partitioning against PE (580 + 510 mL). The solvent was evaporated *in vacuo* to afford the aqueous methanol partition.

Beatrix Bay Shallow

The sponge was blended with MeOH (320 mL) and stirred overnight. The slurry was then stirred with Celite (100 g) wet with 4:1 MeOH/H_2O (200 mL), filtered and re-extracted using the amounts detailed for the Akaroa deep sample. The MeOH/ H_2O ratio was adjusted with the addition of H_2O (150 mL) before partitioning against PE (560 + 500 mL). The aqueous methanol extract was taken to dryness *in vacuo*.

Beatrix Bay Deep

The sponge was split into thirds, and each third blended with MeOH (400 mL). The combined sponge slurry was stirred overnight. Celite (320 g) wet with 1:1 MeOH/H_2O (640 mL) was added to the slurry and stirring continued for 30 minutes before filtering. The sponge residue was extracted with MeOH (6 × 800 mL), stirring for one hour before each filtration. The combined filtrates were divided into two batches. Batch one (3 L) and batch two (2.65 L) were partitioned against PE (600 + 525 mL and 530 + 460 mL respectively). The aqueous methanol partition was concentrated *in vacuo* to provide an aqueous extract (1 L). The aqueous extract was partitioned against EtOAc (4 × 300 mL). Combination of the organic phases followed by evaporation *in vacuo* afforded the EtOAc extract (923 mg).

Each of the above extracts was coated onto Celite (4.18 g). The coated Celite was packed onto the head of a reverse phase (C18) column (100 g, 30 mm × 600 mm) equilibrated to 1:1 MeOH/H_2O. Step gradient elution, under "flash" conditions, of 1:1 MeOH/H_2O to MeOH (200 mL/fraction) followed by a 1:1 MeOH/CH_2Cl_2 fraction and finally CH_2Cl_2 and MeOH strips (400 mL combined) afforded eight fractions. These fractions were assayed against the P388 cell line.

Results (**% MeOH**, mass (mg), *IC_{50} (ng/mL)*).

BB shallow: **50**, ?, > *12500*; **60**, 2886, > *12500*; **70**, 78.2, > *1250*; **80**, 58.9, *832*; **90** 44.4, ≤*9.7*; **100**, 22.2, *882*; **50**, 11.7, > *1250*; **strip**, 9.1, *1146*.

BB mid depth: **50**, ?, *5872*; **60**, 45.1, > *12500*; **70**, 45.5, > *1250*; **80**, 34.8, > *1250*; **90** 46.1, < *9.7*; **100**, 33.8, > *1250*; **50**, 20.3, > *1250*; **strip**, 5.6, > *1250*.

BB deep: **50**, 353.5, > *12500*; **60**, 74.0, > *12500*; **70**, 102.5, > *1250*; **80**, 93.0, *493*; **90** 93.5, ≤*9.7*; **100**, 174.0, > *1250*; **50**, 37.4, *1050*; **strip**, 6.0, > *1250*.

AK shallow: **50**, ?, > *12500*; **60**, ?, > *12500*; **70**, 662.3, > *1250*; **80**, 325.4, *414*; **90** 24.8, *184*; **100**, 17.7, *218*; **50**, 10.9, *292*; **strip**, 4.7, *212*.

AK deep: **50**, ?, > *12500*; **60**, ?, > *12500*; **70**, 83.5, > *1250*; **80**, 41.8, *338*; **90** 15.6, *36*; **100**, 13.1, *720*; **50**, 5.5, *452*; **strip**, 3.1, *935*.

The 90% MeOH/H_2O fraction from each of the BB samples, and the last five fractions (80% MeOH/H_2O onwards) from the AK samples were subjected to size exclusion chromatography on LH20. The LH20 (100 g) was preswollen in CH_2Cl_2 overnight. The column (600 mm × 30 mm) was eluted with CH_2Cl_2 at *ca* 1 mL/min. Nine fractions (50 mL) were collected. At the completion of each column, the LH20 was emptied into a Buchner funnel, washed with MeOH and oven dried (60 °C) overnight. The fractions were dried *in vacuo* and assayed against the P388 cell line.

Results (**fraction**, mass (mg), *IC_{50} (ng/mL)*).

BB shallow: **80.1**, 1.8, *42.7*; **80.2**, 1.7, *3*; **80.3**, 1.6, > *125*; **80.4**, 1.2, > *125*; **80.5**, 1.0, > *125*; **80.6**, 0.8, > *125*; **80.7**, 1.2, > *125*; **80.8**, 1.1, > *125*; **80.9**, 41.6, *276*.

BB deep: **81.1**, 0.9, *21.2*; **81.2**, 1.8, *1.2*; **81.3**, 1.4, *21.2*; **81.4**, 2.1, > *125*; **81.5**, 1.9, > *125*; **81.6**, 3.0, > *125*; **81.7**, 1.9, > *125*; **81.8**, 1.0, > *125*; **81.9**, 39.8, *465*.

BB extra deep: **79.1**, 2.2, *11.9*; **79.2**, 4.8, *3.6*; **79.3**, 2.7, *3.6*; **79.4**, 2.5, *21.2*; **79.5**, 2.3, *21.2*; **79.6**, 1.8, *10*; **7**, 1.2, > *125*; **79.8**, 1.1, > *125*; **79.9**, 88.8, *115*.

AK shallow: **82.1**, 3.5, *18.9*; **82.2**, 4.4, *125*; **82.3**, 3.0, > *125*; **82.4**, 3.0, > *125*; **82.5**, 1.4, > *125*; **82.6**, 2.1, > *125*; **82.7**, 0.8, > *125*; **82.8**, 1.3, > *125*; **82.9**, 61.5, *720*.

AK extra deep: **78.1**, 2.6, > *125*; **78.2**, 1.7, *57*; **78.3**, 2.2, *114.6*; **78.4**, 1.0, *32*; **78.5**, 0.8, *45*; **78.6**, 0.9, > *125*; **78.7**, 0.8, *7.9*; **78.8**, 1.3, *108*; **78.9**, 77.0, > *1250*.

Analysis of selected fractions from the LH20 columns by reverse phase (C18) HPLC used a mobile phase of 60% CH_3CN/H_2O at 1mL/min.

Purification of 80.2 (BB shallow, LH20 fraction 2)

Fraction 80.2 was dissolved in benzene and applied to a normal phase (DIOL) cartridge (250 mg). The cartridge was eluted with benzene, 1:1 benzene/CH_2Cl_2, 1% MeOH/CH_2Cl_2, 1:9 MeOH/CH_2Cl_2 and MeOH (3 mL each). TLC (DIOL, 1% MeOH/CH_2Cl_2) of the fractions indicated that the non polar contaminants were eluted with benzene and the halichondrins were eluted with 1:1 benzene/CH_2Cl_2 and CH_2Cl_2.

Purification of Active Fractions

The active fractions within each series were combined and further concentrated by chromatography on DIOL. Each DIOL cartridge (250 mg) was eluted with benzene, CH_2Cl_2, 1:9 MeOH/CH_2Cl_2 and 1:1 MeOH/CH_2Cl_2 (6 mL each). The halichondrins were contained in the CH_2Cl_2 fraction of each series.

Analysis for Halichondrin Content

The amount of each of the three halichondrins **1.8**, **1.25** and **1.28** in the CH_2Cl_2 fractions were calculated by comparison of their HPLC chromatogram integrals to that of a standard sample of halichondrin B analysed under the same conditions. The mass of the sample of **1.8** was equivalent to that of a CH_2Cl_2 fraction containing pure halichondrins only. The results are shown in Figure 3.4.2.

Native Sponge Extraction

Native sponge (94K3-1, 500 g) was blended with MeOH in a Waring blender. The sponge slurry was stirred overnight. Celite (100 g) wet with 4:1 MeOH/H_2O (200 mL) was added and stirring continued for a further 30 minutes before filtering. The sponge residue was extracted with further MeOH (400 + 350 + 4 ×300 mL), stirred for one hour and filtered. The MeOH/H_2O ratio was adjusted to 4:1 with the addition of H_2O (550 mL) before partitioning against PE (640 + 560 mL). The solvent was evaporated from

the aqueous methanol partition and the residue coated onto Celite (4.18 g). Reverse phase (C18) "flash" chromatography was carried out as described for the aquacultured sponge extracts. Results (**% MeOH**, mass (mg), *IC_{50} (ng/mL)*) **50**, ?, > *12500*; **60**, ?, > *12500*; **70**, 54.0, *253*; **80**, 32.4, *106*; **90** 21.3, < *9.7, 27.8*; **100**, 37.4, *63*; **50**, 8.5, *310*; **strip**, 2.9, *2250*.

The five fractions eluted with 70% MeOH/H_2O through MeOH/CH_2Cl_2 were combined (153 mg) and applied to the head of an LH20 column (100 g, 600 mm × 30 mm). The LH20 column was eluted with CH_2Cl_2 and eight fractions (50 mL) collected. Results (**fraction**, mass (mg), *IC_{50} (ng/mL)*) **98.1**, 4.3, *8.1*; **98.2**, 1.2, *42.7*; **98.3**, 1.1, *47.9*; **98.4**, 1.0, *105*; **98.5**, 1.1, *105*; **98.6**, 0.7, > *125*; **98.7**, 0.8, > *125*; **98.8**, 1.2, > *125*.

Fractions 98.1, 98.2 and 98.3 were combined and further purified by LH20 chromatography. The LH20 column (4g, 880 × 10 mm) was eluted with CH_2Cl_2 and a 20 mL fraction collected, followed by a further four fractions (2 mL each). Halichondrins were identified in the first fraction by TLC (DIOL, 1% MeOH/CH_2Cl_2). This halichondrin content of this fraction was determined, as described for the aquaculture samples, by comparison of the integrals of the halichondrin peaks in the HPLC chromatogram of this fraction with those of standard samples.

7.4 Work Described in Chapter Four

7.4.1 Reaction of Isohomohalichondrin B (1.28) with PMSI (4.1)

A solution of **1.28** (2.5 mg) and **4.1** (0.7 mg) in dry CH_2Cl_2 (200 μL) was stirred at room temperature for 8 hours. TLC (DIOL, 3% MeOH/CH_2Cl_2) of the reaction mixture indicated the starting material was intact. The reaction was continued for a further 19 hours at which time analysis by TLC showed that little reaction had occurred. A ^1H NMR spectrum of the reaction mixture contained resonances belonging to both intact starting material and intact PMSI. The reaction was allowed to continue in the NMR

tube (*ca* 150 μL $CDCl_3$ with 0.1% C_5D_5N) for 4 days. The loss of the characteristic H54/54' and H55 triplet resonances in the ^1H NMR spectrum of the reaction mixture after this time indicated that reaction of **1.28** had occurred. There was little PMSI present. A shift in the resonances of the styryl protons of PMSI from δ_H 6.61 and δ_H 6.43 to δ_H 5.92 and δ_H 7.18 was consistent with formation of the carbamate product. The product was purified on LH20 (1 g), eluted with CH_2Cl_2. Fractions containing the product were identified by TLC (DIOL, 3% MeOH/CH_2Cl_2) and combined as REL3 41.1 (**4.3**, 2.0 mg). ^1H NMR and ^{13}C NMR data are located in Table 4.2.1 and Table 4.2.2.

7.4.2 Reaction of Homohalichondrin B (1.25) with PMSI (4.1)

Homohalichondrin B (**1.25**, 1.3 mg) was treated with **4.1** (0.28 mg) in $CDCl_3$ (150 μL) for 5 days at room temperature. The reaction mixture was separated into 4 fractions with reverse phase (C18) HPLC using 60% CH_3CN/H_2O as the mobile phase (REL3 51.2, 0.4 mg; REL3 51.3, 0.1 mg; REL3 51.4, 0.3 mg; REL3 51.5, < 0.1 mg).

REL3 51.2 was identified as the starting material by comparison of retention time and ^1H NMR spectrum to that of an authentic sample of homohalichondrin B.

REL3 51.3 HRFABMS MH^+ 1363.6353 ($C_{74}H_{95}O_{22}N_2$ requires 1363.6376)

REL3 51.4 HRFABMS MH^+ 1363.6386 ($C_{74}H_{95}O_{22}N_2$ requires 1363.6376)

REL3 51.5 HRFABMS MH^+ 1603.6864 ($C_{87}H_{103}O_{25}N_4$ requires 1603.6911)

7.4.3 Reaction of Homohalichondrin B (1.25) with PMPI (4.2)

Homohalichondrin B (**1.25**, 0.9 mg) and **4.1** (0.17 mg) were dissolved in $CDCl_3$ (*ca* 150 μL) and transferred to a 3 mm NMR tube. The ^1H NMR spectrum of the reaction mixture after 64 hours showed a small amount of **4.1** was still present. The complicated nature of the methyl region of the spectrum indicated a mixture of products had been

formed. Reaction for a further 24 hours had no affect on the appearance of the ^{1}H NMR spectrum of the mixture.

Semipreparative reverse phase (C18) HPLC using a mobile phase of 60% CH_3CN/H_2O was performed on the product mixture. A series of 5 50 μL injections were made, and the peaks eluting at *ca* 290 s (REL3 46.2, **1.25**, 0.2 mg), *ca* 510 s (REL3 46.3), *ca* 600 s (REL3 46.4, **4.7**, 0.7 mg) and 1200 s (REL3 46.5) were collected.

The retention time of **1.25** was characteristic of the starting material. The identity of this fraction was confirmed by comparison of its ^{1}H NMR spectrum with that of an authentic sample of **1.25**. The ^{1}H NMR spectrum of the major component **4.7** contained a doublet of doublets at δ_H 6.2 and δ_H 6.4. The characteristic maleimide proton resonance (δ_H 6.8) was barely visible. This indicated addition to the maleimide olefin had occurred. Reinjection of **4.7** under the same HPLC conditions employed for separation of the reaction mixture gave a reduction in the retention time of **4.7** from 600 s to 470 s. This increase in polarity would be consistent with the addition of methanol to the maleimide olefin.

Compound **4.7** HRFABMS MNa^+ 1391.6292 ($C_{73}H_{96}N_2O_{23}Na$ requires 1391.6301).

Homohalichondrin B (**1.25**, 2.5 mg) and **4.2** (0.48 mg) was transferred to a 3 mm NMR tube in $CDCl_3$ (*ca* 150 μL). The progress of the reaction was monitored by ^{1}H NMR spectroscopy. A ^{1}H NMR spectrum of the reaction mixture after 5 minutes indicated that reaction had occurred, as the methyl region had increased in complexity indicating multiple product formation. No further changes were observed in ^{1}H NMR spectra run after this time.

Semipreparative reverse phase (C18) HPLC was used to separate the reaction mixture into 4 fractions (REL3 48.2-5). The fractions were dried *in vacuo* and transferred to weighed vials in CH_2Cl_2 to avoid reaction of the maleimide.

REL3 48.3 (**4.10**)

Partial ^{1}H and ^{13}C NMR data cited in Table 4.3.4 and Table 4.3.5 respectively.

REL3 48.4 (**4.9**)

Partial ^{1}H and ^{13}C NMR data cited in Table 4.3.2 and Table 4.3.3 respectively. HRFABMS MH^{+} 1337.6229 ($C_{72}H_{93}O_{22}N_2$ requires 1337.6219).

REL3 48.5 (**4.8**)

Partial ^{1}H NMR data cited in Table 4.3.1.

7.4.4 Reaction of Halichondrin B (1.8) with PMPI (4.2)

Halichondrin B (**1.8**, 1.5 mg) was treated with **4.1** (0.29 mg) in $CDCl_3$ (150 μL) for 72 hr. The ^{1}H NMR spectrum of the reaction mixture indicated that at least 3 products had been formed, with the observation of three maleimide proton resonances.

The reaction mixture was separated by reverse phase (C18) HPLC using 60% CH_3CN/H_2O as the mobile phase. Six fractions were collected (REL3 54.2, 229 s; REL3 54.3, 285 s, **4.12**; REL3 54.4, 346 s; REL3 54.5, 374 s, **4.11**; REL3 54.6, 467-523 s; REL3 54.7, 692 s).

REL3 54.2 (**1.8**, 1.1 mg) was identified as starting material by a comparison of its ^{1}H NMR spectrum with that for an authentic sample of halichondrin B.

REL3 54.3 (**4.12**)

Isolated as a colourless oil (< 0.1 mg). HRFABMS MH^{+} 1325.62377 ($C_{71}H_{93}O_{22}N_2$ requires 1325.6219).

REL3 54.5 (**4.11**)

Isolated as a colourless oil (0.4 mg). HRFABMS MH^+ 1325.6229 ($C_{71}H_{93}O_{22}N_2$ requires 1325.6219).

7.4.5 Formation of Norhomohalichondrin B Aldehyde (4.13)

Homohalichondrin B (**1.25**, 0.7 mg) dissolved in MeOH (2 drops) was stirred with aqueous sodium periodate ($NaIO_4$, 150 μL, 5 mM) for 16 hours at room temperature. TLC (DIOL, 5% MeOH/CH_2Cl_2) was used to monitor the reaction. The reaction mixture was extracted with CH_2Cl_2 (4 × 0.5 mL) and the solvent removed to give 0.3 mg of the product (**4.13**). The reaction mixture was reextracted with CH_2Cl_2 to give 0.7 mg of combined **4.13**. A doublet resonance at δ_H 9.70 in the ^1H NMR spectrum of **4.13** confirmed aldehyde formation.

A further batch of **1.25** was cleaved with periodate to provide enough material for thorough assignment of the ^1H and ^{13}C NMR resonances of **4.13**, and for reaction with **4.15**. Homohalichondrin B (**1.25**, 2.9 mg) dissolved in MeOH (5 drops) was stirred with aq. $NaIO_4$ (200 μL, 30 mM) at room temperature for 17 hours. The reaction mixture was extracted with EtOAc and the solvent removed *in vacuo* to give **4.13** as an oil (2.4 mg).

^1H and ^{13}C NMR spectral data are shown in Table 4.4.1 and Table 4.4.2 respectively. HRFABMS MCs^+ 1223.4560 ($C_{60}H_{82}O_{18}Cs$ requires 1223.4552).

7.4.6 Reaction of Norhomohalichondrin B Aldehyde (4.13) with EMCH (4.15)

Sodium acetate (10 μL, 0.18 M) and **4.15** (10 μL, 9.2 mM) were added to **4.13** (1 mg) in MeOH (100 μL). The mixture was mixed thoroughly by vortex and left to sit at room temperature for 17 hours. The reaction mixture was freeze dried to give **4.16**.

HRFABMS MNa^+ 1320.6415 ($C_{70}H_{95}O_{20}N_3Na$ requires 1320.4602). ^1H NMR and ^{13}C NMR spectral data are cited in Table 4.5.1 and Table 4.5.2.

7.4.7 Reduction of Norhomohalichondrin B Aldehyde (4.13)

Norhomohalichondrin B (**4.13**, 3.9 mg) was stirred with $NaBH_4$ (6.8 mg) in IPA (300 μL) for 45 minutes at room temperature. The reaction was quenched with aq. NH_4Cl, diluted with H_2O and applied to a reverse phase (C18) cartridge. The cartridge was washed thoroughly with H_2O before stripping the product off with MeOH (**4.17**, 3.8 mg).

HRFABMS (NIH, magic bullet/CsI) MCs^+ 1225.4703 ($C_{60}H_{84}O_{18}Cs$ requires 1225.4709). ^1H and ^{13}C NMR spectral data are listed in Table 4.6.1 and Table 4.6.2 respectively.

7.4.8 Reaction of Norhomohalichondrin B (4.17) with PMSI (4.1)

Norhomohalichondrin B (**4.17**, 2.4 mg) and PMSI (1.0 mg) were dissolved in $CDCl_3$ (*ca* 150 μL) and transferred to a 3 mm NMR tube. The reaction was monitored by ^1H NMR spectroscopy, and deemed to be complete when the integral of the newly formed styryl proton (δ_H 5.95) was comparable to that of the halichondrin protons H18' and H32. The solvent was evaporated under a stream of N_2 and the residue dissolved in CH_2Cl_2. The residue solution was applied to a small (1 g) LH20 column. The column was eluted with CH_2Cl_2 with the collection of 30 fractions. The fractions containing purely halichondrin material were identified by TLC (DIOL, 4% MeOH/CH_2Cl_2) and combined to give the product (2.5 mg, **4.19**).

7.5 Work Described in Chapter Five

7.5.1 Hydroboration-oxidation

β-Pinene (5 mg) and 9-BBN (5.4 mg) in THF (200 μL) were stirred at 30°C for 18 hours. A drop of water was added to destroy unreacted 9-BBN before the addition of NaOH (49 μL; 1 M) and 50% H_2O_2 (14.5 μL). The reaction mixture was stirred for a further 2 hours at room temperature. The aqueous layer was saturated with K_2CO_3 and the product extracted with CH_2Cl_2.

Homohalichondrin B (**1.25**, 6.2 mg) was stirred with pyridine (200 μL) and acetic anhydride (200 μL) for 16 hours. The reaction mixture was diluted with water and extracted with EtOAc (× 3). The solvent was removed *in vacuo* to give the diacetate **5.3** (6.0 mg). HRFABMS MH^+ 1207.6078 ($C_{65}H_{91}O_{21}$ requires 1207.6052). The ^1H NMR spectrum of the product was comparable to that of an authentic sample of **5.3**.

9-BBN (20 μL; 0.027 M) was added to a stirred solution of **5.3** (0.6 mg) in THF (180 μL). The reaction was stirred for 8 hours at 30°C before quenching with a drop of water. NaOH (5.5 μL; 0.1 M) and 50% H_2O_2 (17 μL; 0.1 M) were added and stirring continued for a further 2 hours. The mixture was diluted with water and extracted with EtOAc (× 3). A ^1H NMR spectrum of the organic extract showed that the homohalichondrin B diacetate (**5.3**) was still intact.

The reaction was repeated using the conditions described above. The hydroboration step was allowed to continue for 5 days before treatment with basic hydrogen peroxide. A ^1H NMR spectrum of the organic extract showed that the exocylic methylene protons remained intact.

Homohalichondrin B diacetate (**5.3**, 0.5 mg) was treated with 9-BBN dimer (1.0 mg) in THF (50 μL) for 88 hours before quenching the reaction with ethanol. NaOH (82 μL; 0.1 M) and 50% H_2O_2 (167 μL; 0.147 M) were added (dropwise) and the mixture stirred

for 6 hours before extracting with EtOAc (× 3). A ^{1}H NMR spectrum of the extract showed that the diacetate **5.3** was still intact.

Borane dimethyl sulfide (2.0 M solution in toluene) was used neat or as a dilute solution.

$Me_2S.BH_3$ (1.5 μL; 0.1 M) and $CDCl_3$ (50 μL) were added to homohalichondrin B diacetate (0.5 mg) in dry $CDCl_3$ (100 μL) in an NMR tube filled with argon. The reaction was monitored half hourly by ^{1}H NMR spectroscopy, and when no change was observed an aliquot of $Me_2S.BH_3$ solution (2 μL) was added. Three such additions were made with no change in the ^{1}H NMR spectrum of the starting material. Freshly prepared $Me_2S.BH_3$ (25 μL; 0.1 M) was added to the reaction solution to ensure a considerable excess of reagent was present. After 16 hours EtOH (10 μL) was added, followed by NaOH (10 μL; 0.1 M) and 50% H_2O_2 (20 μL; 0.147 M). The oxidation was continued for 4 hours before the solvent was removed *in vacuo*. The residue was dissolved H_2O and EtOAc and extracted with EtOAc (3 × 0.5 mL).

$Me_2S.BH_3$ solution (4.2 μL; 0.1 M) was added to **5.3** (0.5 mg) in dry THF (100 μL) and stirred for 16 hours. Ethanol (100 μL), NaOH (14 μL; 0.1 M) and 50% H_2O_2 (29 μL; 0.147 M) were added to the reaction in this order. Stirring was continued for 6 hours before extraction with EtOAc (3 × 0.5 mL). Hydroboration/oxidation of the extract was repeated using the aforementioned conditions to complete the derivatisation of the 19 methylene group.

Benzoic anhydride (4.7 mg), a few crystals of DMAP, and dry pyridine (200 μL) were added to homohalichondrin B (0.3 mg) and stirred for 42 hours before extraction with EtOAc (3 × 0.5 mL). The solvent was removed and the extract dissolved in water and transferred to a reverse phase (C18) cartridge (100 mg). The cartridge was washed with H_2O, eluted with MeOH/H_2O (1:1) to remove the DMAP and stripped with MeOH to give the dibenzoate **5.4** (0.4 mg). ^{1}H NMR ($CDCl_3$ + 0.1% CD_5N) δ 8.05 (m, 2H, ArH),

7.97 (m, 2H, ArH), 7.48-7.57 (m, 2H, ArH), 7.22-7.47 (m, 4H, ArH), 4.55-4.65 (m, 3H, H53/H55/H55'), 5.56 (m, 1H, H54), 0.80-5.05 (remaining halichondrin protons).

Dibenzoate **5.4** was treated with a 4:1 MeOH/H_2O solution of 1% K_2CO_3 (100 μL) and the progress of the reaction monitored by TLC (DIOL, 3% MeOH/CH_2Cl_2). After 24 hours TLC of the reaction mixture gave a single spot of equivalent R_f to that of **1.25**, indicating that cleavage of the protecting groups was complete. The reaction mixture was diluted to a final volume of 1 mL with water before transferring to a reverse phase (C18) cartridge (100 mg). The cartridge was washed with water (3 mL) and 10% MeOH/H_2O (3 mL) before stripping with MeOH to give the diol **1.25**.

Homohalichondrin B (**1.25**, 1 mg) was protected as the dibenzoate as described above. Homohalichondrin B dibenzoate (**5.4**, 1.0 mg) in THF (100 μL) was treated with a THF solution of borane dimethyl sulfide (8.4 μL; 0.2 M). The reaction was stirred for 17 hours. A few drops of ethanol were added followed by NaOH (28 μL; 0.1 M) and H_2O_2 (58 μL; 0.147 M). Stirring was continued for a further 6 hours before extracting with EtOAc. Analysis of the extract by TLC (DIOL, 3% MeOH/CH_2Cl_2) showed that there was a mixture of products. Semipreparative reverse phase (C18) HPLC using a mobile phase of 60% CH_3CN/H_2O was used to separate the mixture into seven fractions. Five of these fractions (REL3 88.1 (0.1 mg), REL3 88.2 (< 0.1 mg), REL3 88.5 (0.3 mg, **5.5**), REL3 88.6 (0.2 mg) and REL3 88.7 (0.1 mg)) had ^{1}H NMR spectra containing halichondrin-type resonances. HRFABMS MCs^+ 1499.5597 ($C_{75}H_{98}O_{23}Cs$ requires 1499.5550).

Homohalichondrin B (**1.25**, 1.2 mg) in pyridine (25 μL) was stirred with TBDPSCl (25 μL) for 42 hours. The reaction mixture was diluted with toluene/hexanes (1:1) and transferred to a normal phase (DIOL) cartridge (100 mg). The cartridge was eluted toluene/hexanes (1:1), toluene, toluene/CH_2Cl_2 (1:1) and CH_2Cl_2 (5 mL of each). Halichondrin-type resonances were observed in the ^{1}H NMR spectrum of the fractions eluted with toluene and toluene/CH_2Cl_2. TLC (DIOL, 3% MeOH/CH_2Cl_2) indicated that

these fractions contained a mixture of halichondrin-derived components. Attempts to purify the product mixture using reverse phase (C18) HPLC were unsuccessful.

7.5.2 Osmylation

7.5.2.1 Osmylation of **5.3**

Homohalichondrin B diacetate (**5.3**, 3.4 mg) in pyridine (60 μL) was treated with an ethereal solution of OsO_4 (17.2 μL; 0.2 M). The reaction mixture was stirred at room temperature for 4.5 hours. Sodium metabisulfite (5.4 mg) in 60% pyridine/water was added and stirring continued for 25 minutes. The product mixture was obtained by extraction with CH_2Cl_2 (4×0.5 mL). TLC (DIOL, 3% MeOH/CH_2Cl_2) showed that the extract contained two halichondrin-like components. The mixture was chromatographed on DIOL (1 g in a Pasteur pipette), eluting with a gradient starting at hexanes/toluene (1:1) through toluene to CH_2Cl_2. Thirty six fractions (0.5 mL) were collected (REL3 105.1-REL3 105.36). ^{1}H NMR spectroscopy was used to identify the fractions containing halichondrin-type compounds. Fractions REL3 105.25 - REL3 105.34, eluted with CH_2Cl_2, were combined as the diol **5.6** (1.2 mg).

^{1}H and ^{13}C NMR spectral data are listed in Table 5.3.1 and Table 5.3.2 respectively. HRFABMS MCs^+ 1373.5022 ($C_{65}H_{92}O_{23}Cs$ requires 1373.5080).

The remaining halichondrin-containing fractions (REL3 105.35-REL3 105.43) were combined for re-osmylation. The combined fraction (1.5 mg) was stirred with an ethereal solution of OsO_4 (13 μL; 0.2 M) in pyridine (60 μL) for 4.5 hours. Sodium metabisulfite (1 mg) dissolved in pyridine (60 μL) and H_2O (80 μL) was added and the reaction stirred for a further 20 minutes. The reaction mixture was extracted with EtOAc (4 × 0.5 mL) to give the product **5.7** (1.4 mg). Refer to Section 7.5.2.3 for spectral data.

7.5.2.2 Protection of **1.25**

1.25 (14.4 mg) was stirred with acetic anhydride (100 μL) and pyridine (100 μL) for 16 hours at room temperature. The reaction was quenched with water and extracted with EtOAc to give **5.3** (15.4 mg). The ^{1}H NMR spectrum of **5.3** was comparable to that of an authentic sample.

7.5.2.3 Further Osmylation of **5.3**

Homohalichondrin B diacetate (**5.3**, 15.4 mg) in pyridine (100 μL) was treated with an ethereal solution of OsO_4 (72 μL; 0.2 M) for 4.5 hours at room temperature. Sodium metabisulfite (24.3 mg) dissolved in pyridine (100 μL) and H_2O (150 μL) was added and the reaction stirred for a further 20 minutes before extracting with EtOAc (4 × 0.5 mL). The product mixture was chromatographed on DIOL (30 g, 15 × 350 mm). A gradient from toluene to CH_2Cl_2 was used to elute the column, with 26 fractions collected (REL3 111.1-REL3 111.26). ^{1}H NMR spectroscopy was used to identify fractions containing halichondrin-type compounds (REL3 111.7-REL3 111.25). REL3 111.14-16 were combined on the basis of their identical ^{1}H NMR spectra to give **5.7** (3.4 mg).

^{1}H and ^{13}C NMR spectral data are shown in Table 5.3.3 and Table 5.3.4 respectively. HRFABMS MH^+ 1275.6148 ($C_{65}H_{95}O_{25}$ requires 1275.6162).

REL3 111.8 and REL3 111.9 were combined as REL3 112.1 (3.2 mg). HRFABMS of this combined fraction gave a parent ion MH^+ 1241.6124 ($C_{65}H_{93}O_{23}$ requires 1241.6107), isobaric to the diol **5.6**.

7.5.2.4 Periodate Cleavage of **5.6**

Compound **5.6** (0.9 mg) was dissolved in MeOH (2 drops) before adding H_2O (100 μL) and aqueous $NaIO_4$ (22 μL; 40 mM). The reaction mixture (milky) was stirred for 15 hours and then extracted with EtOAc.

7.5.2.5 Periodate Cleavage of REL3 112.1

REL3 112.1 (3.2 mg) was dissolved in MeOH (~ 4 drops) before adding H_2O (100 μL) and aqueous $NaIO_4$ (78 μL; 40 mM). The reaction was stirred for 16 hours before extracting with EtOAc (4 × 0.5 mL).

Analytical reverse phase (C18) HPLC (mobile phase 60% CH_3CN/H_2O) was used to identify three halichondrin-like components (retention time 419 s; 595 s 1340s) in the organic extracts from both of the above cleavage reactions. Semipreparative reverse phase (C18) HPLC with a mobile phase of 80% CH_3CN/H_2O was used to separate the three components **5.8** (2.0 mg), **5.9** (0.8 mg) and **5.10** (~ 0.1 mg).

The ^1H and ^{13}C NMR spectral data for **5.8** are listed in Table 5.3.5 and Table 5.3.6. HRFABMS MCs^+ 1341.4834 ($C_{64}H_{88}O_{22}$ requires 1341.4818).

The ^1H and ^{13}C NMR spectral data for **5.9** are listed in Table 5.3.9 and Table 5.3.10. HRFABMS MCs^+ 1373.5104 ($C_{65}H_{92}O_{23}$ requires 1373.5080).

The ^1H and ^{13}C NMR spectral data for **5.10** are listed in Table 5.3.7 and Table 5.3.8. HRFABMS MCs^+ 1341.4832 ($C_{64}H_{88}O_{22}$ requires 1341.4818).

7.5.2.6 Periodate Cleavage of **5.7**

Compound **5.7** (4.8 mg) was dissolved in a few drops of MeOH before adding H_2O (100 μL) and aqueous $NaIO_4$ (113 μL; 40 mM). The reaction was stirred for 15 hours before

extracting with EtOAc (3 × 0.5 mL). The solvent was evaporated *in vacuo* to give the diketone **5.1** (4.5 mg).

The ^{1}H and ^{13}C NMR spectral data have been assigned previously.[95] HRFABMS (NIH; magic bullet/CsI) MCs^{+} 1343.4602 ($C_{63}H_{86}O_{23}Cs$ requires 1343.4611).

7.5.2.7 Deprotection of **5.6**

Compound **5.6** (0.3 mg) was treated with a 4:1 MeOH/H_2O solution of 1% K_2CO_3 (100 μL) for 22 hours. The reaction mixture was diluted with water and extracted with EtOAc (4 × 0.5 mL).

Compound **5.6** (0.3 mg) was treated with K_2CO_3 as described above for 72 hours. The reaction mixture was diluted with water and the product extracted with EtOAc (4 × 0.5 mL).

HRFABMS MCs^{+} 1289.4858 ($C_{61}H_{88}O_{21}Cs$ requires 1289.4869). LRFABMS *m/z* 1307.3 (MCs^{+}+18), *m/z* 1321.7 (MCs^{+}+32).

7.5.3 Methylenation

7.5.3.1 Preparation of Lombardo's Reagent

Lombardo's Reagent was prepared according to a literature procedure.[104] The use of freshly purified zinc dust was critical to the successful synthesis of the reagent.

7.5.3.2 Methylenation of Cholestanone

Ice cold Lombardo's reagent (1 mL) was added dropwise to a stirred solution of cholestanone (10 mg) in THF (2 mL). Stirring was continued for 5 minutes upon completion of reagent addition. The reaction was quenched with saturated $NaHCO_3$ and the product extracted into diethyl ether, washed with brine and dried over anhydrous

$MgSO_4$. ^1H NMR ($CDCl_3$) δ 4.54 (m, 2H, $=CH_2$), 0.70-2.25 (m, 43H), 0.65 (s, 3H, CH_3).

7.5.3.3 Methylenation of Diketone **5.1**

Ice cold Lombardo's reagent (1 mL) was added dropwise to a stirred solution of **5.1** (1.5 mg) in THF (2 mL). Stirring was continued for 5 minutes upon completion of reagent addition. The reaction was quenched with saturated $NaHCO_3$ and the product extracted into diethyl ether, washed with brine and dried over anhydrous $MgSO_4$.

7.6 Work Described in Chapter 6

7.6.1 Reductive Amination

7.6.1.1 Cholestanone and Phenethylamine Hydrochloride

Cholestanone (2.0 mg), phenethylamine hydrochloride (5.7 mg), sodium acetate (4.2 mg) and a solution of acetic acid in MeOH (166 μL; 17.7 mM) were stirred for 30 minutes at room temperature before the addition of $NaCNBH_3$ (20 μL; 0.26 M). The reaction was stirred for a further 64 hours. The solvent was evaporated, and the CH_2Cl_2 soluble material applied to a Pasteur pipette packed with DIOL. The column was eluted with CH_2Cl_2, CH_2Cl_2/EtOAc (1:1) and EtOAc (4mL of each). The product was eluted in the first two fractions. ^1H NMR ($CDCl_3$) δ 7.34-7.18 (m, 5H), 3.05-2.89 (m, 2H), 2.00-0.82 (m, 14H), 0.78 (s, 3H), 0.63 (s, 3H). HRFABMS (NIH, mb/peg/ef) MH^+ 492.4537 ($C_{35}H_{58}N$ requires 492.4569).

7.6.1.2 Cholestanone and Ammonium Acetate

Cholestanone (2.0 mg), ammonium acetate (4 mg) and acetic acid (167 μL; 1.75 mM) were stirred at room temperature for 30 minutes before the addition of $NaCNBH_3$ (20

μL; 0.26 M). The reaction was stirred at room temperature for a further 68 hours. The solvent was evaporated and the residue dissolved in H_2O and applied to a reverse phase (C18) cartridge. The cartridge was washed with H_2O (4 mL) and stripped with MeOH and CH_2Cl_2. These fractions gave a positive (bright orange spot) result when visualised with Dragendorff's spray reagent on a silica TLC plate.

7.6.1.3 Norhomohalichondrin B Aldehyde (**4.13**) and Ammonium Acetate

Norhomohalichondrin B aldehyde (**4.13**, 0.5 mg), ammonium acetate (10 μL; 0.45 M) and acetic acid (8 μL; 0.026 mM) were stirred for 15 minutes before adding $NaCNBH_3$ (2 μL; 0.23 M). The reaction was stirred at room temperature for 65 hours before evaporating the solvent. The complex nature of the methyl region indicated more than one product had been formed. The mixture was separated by reverse phase (C18) HPLC into five fractions using 55% CH_3CN/H_2O as the mobile phase. There was insufficient material recovered for further analysis by ^1H NMR spectroscopy.

Norhomohalichondrin B aldehyde (**4.13**, 0.6 mg), ammonium acetate (10 μL; 0.54 M) and acetic acid (200 μL; 0.093 M) were stirred with crushed activated 3Å molecular sieves for 30 minutes before the addition of $NaCNBH_3$ (10 μL; 0.11 M). The reaction was stirred at room temperature for a further 65 hours. The solvent was removed *in vacuo* and the residue analysed by reverse phase (C18) HPLC, using a mobile phase of 60% CH_3CN/H_2O.

7.6.1.4 Oxidation of Citronellol

Dess-Martin periodinane (1.75 g) and citronellol (500 μL) in CH_2Cl_2 (40 mL) were stirred at room temperature until TLC (SiO_2, 3:2 PE/EtOAc, vis. with $KMnO_4$) indicated that the reaction was complete (1.5 h). The reaction was diluted with Et_2O (40 mL) and poured into saturated $NaHCO_3$ containing a 7-fold excess of sodium persulfate. The aqueous layer was extracted with Et_2O and the combined organic layers washed with

$NaHCO_3$, brine, dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification of the residue by flash SiO_2 chromatography (4:1 PE/EtOAc) yielded citronellal (223 mg). ^1H NMR ($CDCl_3$) δ 9.74 (t, 1H), 5.07 (m, 1H) 2.44-1.15 (m, 13H) 0.96 (d, 3H).

7.6.1.5 Citronellal and Ethylenediamine Hydrochloride

Ethylenediamine hydrochloride (2.7 M) was formed by combining ethylenediamine dihydrochloride (1.99 g) slurried in MeOH (10 mL) with ethylenediamine (1 mL).

Ethylenediamine hydrochloride (100 μL; 2.7 M) and citronellal (5 μL) were stirred with $NaCNBH_3$ (100 μL; 0.28 M) at room temperature. The reaction was monitored by TLC (SiO_2, 3:2 PE/EtOAc, vis. with $KMnO_4$). After 17 hours the reaction was basified with Na_2CO_3, extracted with EtOAc, dried over $MgSO_4$ and the solvent evaporated to give a sweetly scented oil. GCMS indicated that the major component was the reductive amination product (**6.2**). A minor component was identified as the dimer of **6.2**.

7.6.1.6 Norhomohalichondrin B Aldehyde (**4.13**) and Ethylenediamine Hydrochloride

Norhomohalichondrin B aldehyde (**4.13**, 1.0 mg) and ethylenediamine hydrochloride (5 μL) in MeOH (200 μL) were stirred at room temperature for 30 minutes before the addition of $NaCNBH_3$ (10 μL; 0.095 M). The reaction was stirred for 65 hours before evaporating the solvent. Additional resonances were observed in the region of δ_H 2.5-3.0 in the ^1H NMR spectrum of the residue. No useful MS result was obtained.

7.6.1.7 Norhomohalichondrin B Aldehyde (**4.13**) and Sodium Cyanoborohydride

Norhomohalichondrin B aldehyde (**4.13**, 0.5 mg) in MeOH (200 μL) was stirred with $NaCNBH_3$ (5 μL; 0.095 M) at room temperature for 65 hours before the solvent was evaporated. The complex nature of the methyl region in the ^1H NMR spectrum of the residue indicated that more than one halichondrin component was present. LRFABMS

m/z 1113.5 (**4.13**Na^+), 1115.5 (**4.17** Na^+), 1131.5 (**4.17**K^+), 1223.4 (**4.13**Cs^+), 1225.4 (**4.17**Cs^+).

7.6.2 The Azide Route

7.6.2.1 Tosylation of Citronellol

p-Toluenesulfonyl chloride was purified prior to use.[128]

A solution of citronellol (12 μL) and pyridine (11 μL) in CH_2Cl_2 (200 μL) was cooled to 0°C before the dropwise addition of *p*-toluenesulfonyl chloride (TsCl, 25 mg) in CH_2Cl_2 (100 μL). The reaction was stirred for 2 hours at 0°C and at room temperature for 17 hours. The reaction mixture was diluted with H_2O and diethyl ether, washed with 2N HCl, saturated $NaHCO_3$ and H_2O and dried over $MgSO_4$. The solvent was evaporated *in vacuo* to give the product (15.5 mg). ^1H NMR ($CDCl_3$) δ 7.79 (m, 2H), 7.34 (m, 2H), 5.02 (m, 1H), 4.05 (m, 2H), 2.45 (s, 3H), 2.03-1.00 (m, 13H), 0.88 (d, 3H).

7.6.2.2 Formation of Citronellazide

NaN_3 (42.8 mg) in DMF (100 μL) was added to citronellol tosylate (15.5 mg) in DMF (600 μL). The reaction slurry was stirred for 17 hours at room temperature. TLC (SiO_2, 4:1 PE/EtOAc, vis. with $KMnO_4$) showed that the reaction was complete. The reaction mixture was diluted with water and diethyl ether and washed twice with water to remove any unreacted azide. The ether layer was then washed with NH_4Cl, saturated $NaHCO_3$ and brine and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a sweetly scented yellow oil (3.5 mg). ^1H NMR ($CDCl_3$) δ 5.08 (m, 1H), 3.28 (m, 2H), 1.98 (m, 2H), 1.72-1.04 (m, 11H), 0.90 (d, 3H).

7.6.2.3 Futher Tosylation of Citronellol

TsCl (1.38 g) in CH_2Cl_2 (10 mL) was added dropwise to an ice-cold solution of citronellol (1.2 mL), DMAP (103 mg) and diisopropylethylamine (1.26 mL) in CH_2Cl_2 (10 mL). The reaction was stirred for 18 hours, leaving the icebath to adjust to room temperature. The reaction was diluted with CH_2Cl_2 and washed with H_2O (× 2), NH_4Cl, and H_2O, dried ($MgSO_4$) and the solvent evaporated. ^1H NMR indicated a mixture (11:9) of the tosylate and chloride had been formed.

7.6.2.4 Formation of More Citronellazide

The tosylate/chloride mixture was stirred with NaN_3 (4.28 g) in DMF (15 mL) at room temperature for 18 hours. The reaction was diluted with water and extracted with diethyl ether. The ether extract was washed with water (× 2), NH_4Cl, $NaHCO_3$ and brine, dried ($MgSO_4$) and the solvent evaporated to give an orange oil. Flash chromatography on silica with petroleum ether gave citronellazide (309 mg).

7.6.2.5 Reduction of Citronellazide

Hydrogenation of citronellazide (106.6 mg) was carried out with Lindlar catalyst (38.7 mg) in EtOH (40 mL) at room temperature and atmospheric pressure for 2 hours. The reaction was then filtered through Celite and the solvent evaporated to yield citronellamine (91.5 mg). GCMS indicated that citronellamine was the major component (*ca* 95%). HREIMS M^+ 155.1668 ($C_{10}H_{21}N$ requires 155.1674). ^1H NMR ($CDCl_3$) δ 5.07 (m, 1H), 2.69 (m, 2H), 1.96 (m, 2H), 1.71-1.02 (m, 13H), 0.85 (m, 2H).

7.6.3 Halichondrin Reactions

7.6.3.1 Attempted Tosylation of Norhomohalichondrin B (**4.17**)

Norhomohalichondrin B (**4.17**, 0.9 mg), diisopropylethylamine (10 μL; 92 mM) and DMAP (10 μL; 8.2 mM) in CH_2Cl_2 (80 μL) was cooled to 0°C. TsCl in CH_2Cl_2 (100 μL; 8.9 mM) was added dropwise and the reaction stirred for 2 hours (0°C to RT). The reaction was diluted with H_2O and extracted with diethyl ether. The ether layer was washed with NH_4Cl, brine and H_2O, dried ($MgSO_4$) and the solvent evaporated. The ^1H NMR spectrum of the extract indicated that no reaction had occurred.

The reaction was repeated using the starting material recovered above. The quantities of reagents used above were doubled, and the reaction stirred for 2 hours as before. Once more, the ^1H NMR spectrum of the organic extract of the reaction mixture contained resonances belonging to the starting material. The aromatic resonances of TsCl were also observed (δ 7.93, 7.41).

Norhomohalichondrin B (**4.17**, 0.6 mg), DMAP (1 crystal) and diisopropylethylamine (2 μL) in CH_2Cl_2 were cooled to 0°C before the dropwise addition of TsCl (2.0 mg) in CH_2Cl_2 (100 μL). The reaction was stirred for 2 hours (0°C to RT) and then diluted with water and extracted with diethyl ether. The organic extract was washed as described previously, dried ($MgSO_4$) and the solvent evaporated to yield an oily residue (8.5 mg). The ^1H NMR spectrum of the residue showed that contamination had occurred. Base-line halichondrin-type resonances were observed. The source of this contamination was traced to the diethyl ether.

TLC (DIOL, 4% MeOH/CH_2Cl_2) indicated that the contaminated residue contained starting material **4.17**.

7.6.3.2 Tosylation of Homohalichondrin B (**1.25**)

Homohalichondrin B (**1.25**, 1.5 mg), diisopropylethylamine (2.5 μL) and DMAP (1-2 crystals) in CH_2Cl_2 were cooled to 0°C before the dropwise addition of TsCl (2 mg) in CH_2Cl_2 (100 μL). The reaction was stirred for 2 hours (0°C to RT). The reaction was diluted with H_2O and extracted with EtOAc. The EtOAc extract was washed with NH_4Cl, brine and H_2O, dried ($MgSO_4$) and the solvent evaporated. The development of a shoulder on the H47 resonance in the ^1H NMR spectrum of the residue indicated that reaction was beginning to occur. However, the starting material **1.25** was largely intact.

The recovered starting material was reacted as described above. The reaction was stirred for 18 hours before working up as described previously. The H47 resonance was observed upfield δ_H 0.02 relative to that of **1.25**. The absence of the characteristic H55/H55' methylene resonance was consistent with tosylation. HRFABMS MCs^+ 1409.4918 ($C_{68}H_{92}O_{21}SCs$ requires 1409.4903). LRFABMS 1563.5 (homohalichondrin B ditosylateCs^+).

7.6.3.3 Tosylation of Norhomohalichondrin B (**4.17**)

Norhomohalichondrin B (**4.17**, 0.5 mg), diisopropylethylamine (2.5 μL) and DMAP (1 crystal) in CH_2Cl_2 (100 μL) were cooled to 0°C before the dropwise addition of TsCl (2 mg) in CH_2Cl_2 (100 μL). The reaction was stirred for 17 hours (0°C to RT) and then diluted with EtOAc, washed with H_2O, NH_4Cl, brine and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield the product. HRFABMS (NIH, mb/CsI) MCs^+ 1379.4844 ($C_{67}H_{90}O_{20}SCs$ requires 1379.4797).

7.6.3.4 Azide Substitution of Norhomohalichondrin B Tosylate (**6.8**)

Norhomohalichondrin B tosylate (**6.8**, 0.3 mg) was stirred with NaN_3 (51 mg) in DMF (400 μL) at 40°C for 18 hours. Once cooled, the reaction was diluted with H_2O and extracted with EtOAc. The organic extract was washed with H_2O, NH_4Cl, brine and

H_2O, dried ($MgSO_4$) and the solvent evaporated. Two H47 resonances were observed in the ^1H NMR spectrum of the residue (0.4 mg). Analysis of the mixture by reverse phase (C18) HPLC (mobile phase 70% CH_3CN/H_2O) equipped with both diode array and refractive index (RI) detection found that the ratio of components was 4:1 azide:tosylate.

7.6.3.5 Hydrogenation

Hydrogenation of the azide/tosylate mixture (~ 0.4 mg) was carried out with Lindlar catalyst (~ 0.9 mg) in EtOH (1 mL) at room temperature and atmospheric pressure for 5 hours. The reaction was filtered through Celite and the solvent evaporated. The ^1H NMR spectrum of the residue was identical to that of the starting material.

Hydrogenation of the azide/tosylate mixture was undertaken for a further 48 hours using the conditions described above. The exocyclic methylenes remained intact but it was difficult to ascertain if any amine had been formed.

7.6.3.6 "Large-scale" Preparation of Norhomohalichondrin B Azide (**6.9**)

Homohalichondrin B (**1.25**, 44.0 mg) in MeOH (20 μL) was treated with $NaIO_4$ (1 mL; 50 mM). H_2O (500 μL) was added to dissolve the white precipitate. The reaction was stirred for 18 hours at room temperature. The reaction was diluted with H_2O and extracted with EtOAc (4 × 1.5 mL). The solvent was evaporated to give norhomohalichondrin B aldehyde (**4.13**, 43.3 mg).

Norhomohalichondrin B aldehyde (**4.13**, 24.5 mg) and $NaBH_4$ (22.7 mg) in IPA (2 mL) were stirred for 45 minutes. The reaction was quenched with NH_4Cl and extracted with EtOAc. The extract was washed with brine and H_2O, dried ($MgSO_4$) and the solvent evaporated to yield norhomohalichondrin B (**4.17**, 19.3 mg).

Norhomohalichondrin B (**4.17**, 19.1 mg), diisopropylethylamine (7 μL) and DMAP (4.2 mg) in CH_2Cl_2 (150 μL) were cooled to 0°C before the dropwise addition of TsCl (6.7 mg) in CH_2Cl_2 (100 μL). The reaction was stirred for 14 hours. The reaction was diluted with H_2O and extracted with EtOAc. The extract was washed with NH_4Cl, brine and H_2O, dried ($MgSO_4$) and the solvent evaporated to yield the tosylate **6.8** (20.1 mg). HRFABMS (NIH, magic bullet/CsI) MCs^+ 1379.4844 ($C_{67}H_{90}O_{20}SCs$ requires 1379.4797). ^1H and ^{13}C NMR data are listed in Table 6.3.1 and Table 6.3.2.

Norhomohalichondrin B tosylate (**6.8**, 8.5 mg) was stirred with NaN_3 (50 mg) in DMF (400 μL) at 40°C for 19 hours. The reaction was diluted with H_2O and extracted with EtOAc. The EtOAc extract was washed with H_2O, NH_4Cl and brine, dried ($MgSO_4$) and the solvent evaporated. Reverse phase (C18) HPLC (mobile phase 70% CH_3CN/H_2O) showed that the residue was a 7:3 mixture of azide and starting material.

The mixture was stirred with NaN_3 (51.3 mg) in DMF (400 μL) at 40°C. Periodically an aliquot (10 μL) was removed and diluted with CH_3CN (990 μL) for HPLC analysis. After 57 hours the reaction was diluted with water and extracted with EtOAc. The extract was washed with H_2O, NH_4Cl and brine, dried ($MgSO_4$) and the solvent evaporated to yield the azide **6.9** (5.4 mg). HRFABMS (NIH, magic bullet/CsI) MCs^+ 1250.4822 ($C_{60}H_{83}O_{17}N_3Cs$ requires 1250.4773). ^1H and ^{13}C NMR data are shown in Table 6.3.3 and Table 6.3.4.

7.6.3.7 Reduction of Norhomohalichondrin B Azide (**6.9**)

Hydrogenation of norhomohalichondrin B azide (**6.9**, 2.0 mg) was carried out with Lindlar catalyst (1.5 mg) in EtOH (1 mL) at room temperature and atmospheric pressure. The reaction was monitored by reverse phase (C18) HPLC using a mobile phase of 70% CH_3CN/H_2O. Aliquots (10 μL) were removed from the reaction and filtered through a 0.45 μm filter capped with Celite. The solvent was evaporated and the residue dissolved in CH_3CN (80 μL). After 32 hours the reaction was filtered

through Celite and the solvent evaporated. The H54 azide resonance was significantly reduced in the ^{1}H NMR spectrum of the orange residue. The exocyclic methylene resonances were present. The complicated nature of the methyl resonances indicated the presence of more than one halichondrin-type compound. Resonances associated with an unidentified contaminant were observed in the region of δ_H 0.8-0.9.

Reverse phase (C18) chromatography was used to separate the components. Trace amounts of azide **6.9** were recovered, but the remaining halichondrin material was lost.

Hydrogenation of **6.9** (3.4 mg) was carried out with Lindlar catalyst (1.9 mg) in EtOH (1 mL) at room temperature and atmospheric pressure for 41 hours. The reaction was filtered through Celite and the solvent evaporated to yield an orange oil (3.8 mg, **6.10**). HRFABMS (NIH, magic bullet/CsI) MCs^+ 1224.4812 ($C_{60}H_{85}O_{17}NCs$ requires 1224.4868). ^{1}H and ^{13}C NMR data are listed in Table 6.3.5 and Table 6.3.6.

7.6.4 Polymer Reactions

7.6.4.1 Determination of ε_{400} for ONp

Two dilutions were made from each of two solutions of *p*-nitrophenol in phosphate buffered saline (pH 7.68). Duplicate absorbance readings of the six samples were obtained at 400 nm. A (1.858×10^{-4} M) = 2.2672, 2.2653; A (1.4988×10^{-4} M) = 1.9292. 1.9707; A (1.199×10^{-4} M) = 1.5901, 1.5693; A (7.43×10^{-5} M) = 0.96690, 0.96710; A (6.79×10^{-5} M) = 0.90921, 0.84628; A (5.575×10^{-5} M) = 0.72824, 0.69614. ε_{400} 13097 $M^{-1}cm^{-1}$.

7.6.4.2 Trial Reactions

An aliquot (60 μL) of polymer precursor (5.0 mg) in DMSO (1 mL) was diluted to 1 mL with phosphate buffered saline. The absorbance of this diluted solution at 400 nm was

0.1087. The number of moles of free amine in the polymer precursor (5 mg) was calculated as 1.38×10^{-7}.

Polymer precursor (5.0 mg) was stirred with 1-amino-2-propanol (5 μL) and TEA (5 μL) in DMSO (990 μL) for 10 minutes. An aliquot (60 μL) of the reaction solution was diluted to 1 mL with phosphate buffered saline. The absorbance of the diluted solution at 400 nm was 0.7992. This value was used to calculate an estimated ONp content of 2.7 mol%.

1-Amino-2-propanol (5 μL) and TEA (9 μL) was added to polymer precursor (5.0 mg) in DMSO (86 μL). The reaction was stirred at room temperature for 23 hours. An aliquot (6 μL) of the reaction mixture was diluted to 1 mL with phosphate buffered saline and the absorbance at 400 nm measured against a phosphate buffered saline blank (1.1938). This equates to a side chain content of 4.5 mol%. The remaining sample was diluted with MeOH to a total volume of 1 mL and applied to an LH20 column (5 g; 13 × 220 mm). The eluent was run through a single wavelength (206 nm) spectrometer. A fraction collector was used to collect 2 minute fractions of *ca* 1-1.2 mL each. The polymer containing fractions (5-8) were combined for use as a standard. ONp was concentrated in fractions 18-24.

7.6.4.3 Reaction of Norhomohalichondrin B amine (**6.10**) with polymer precursor

Norhomohalichondrin B amine (**6.10**, 1.4 mg) and TEA (10 μL; 0.13 mM) was stirred with polymer precursor (5.0 mg) in DMSO (90 μL) at room temperature for 23 hours. An aliquot of the reaction solution (6 μL) was diluted to 1 mL with phosphate buffered saline. The absorbance of the dilute solution at 400 nm was 0.67325. This equates to the addition of 7.2 mol of **6.10**. 1-Amino-2-propanol (5 μL) was added and the reaction was stirred for a further 16 hours at room temperature. Another aliquot (6 μL) was diluted to 1 mL with phosphate buffered saline and the absorbance recorded at 400 nm (1.2134). The remaining reaction mixture was diluted to 1 mL with MeOH and then

applied to an LH20 column. The column was eluted with MeOH at 0.5 mL/min, and 1 mL fractions collected. TLC (SiO_2, 5% MeOH/CH_2Cl_2) visualised with Dragendorff's spray reagent was used to identify fractions containing free amine (14-16). Fractions containing purely polymer-bound amine (8-13) were combined (4.12 mg).

Appendix 1

1.1 Calculation of side chain content in polymer precursor

Polymer precursor (5.0 mg) was treated with an excess of 1-amino-2-propanol in DMSO (1 mL) for 23 hours. A subsample (60 μL) of the solution was diluted to 1 mL with phosphate buffered saline. The absorbance of this dilute solution was measured at 400 nm.

absorbance of polymer precursor sample	1.1938
corrected absorbance (-0.1087 to account for free ONp in polymer precursor)	1.0851
concentration (absorbance/molar absorbtivity (ε=13097 $M^{-1}cm^{-1}$))	8.29×10^{-5} mol L^{-1}
moles side chain in measured sample (1 mL)	8.29×10^{-8} mol
moles side chain in total sample (used 6 μL from the total sample of 100 μL)	1.38×10^{-6} mol (A)
mass of side chain ($M_{(side\ chain)}$ 581.6 $gmol^{-1}$)	8.03×10^{-4} g
mass of HPMA (5.0 - 8.03×10^{-4} g)	4.20×10^{-3} g
moles of HPMA ($M_{(HPMA)}$ 143.2 $gmol^{-1}$)	2.93×10^{-5} mol (B)
mol% side chain (A/(A+B))	4.5%

[1] Williams, D. H.; Stone, M. J.; Hauck, P. R.; Rahman, S. K. *J. Nat. Prod.*, **1989**, *52*, 1189-1208.

[2] Parkin, D. M.; Läärä, E.; Muir, C. S. *Int. J. Cancer*, **1988**, *41*, 184-197.

[3] de Vries, D. J.; Hall, M. R. *Drug Dev. Res.*, **1994**, *33*, 161-173.

[4] May, R. M. *Science*, **1988**, *241*, 1441-1449.

[5] Garson, M. J. In: *Sponges in Time and Space*, van Soest, R. W. M.; van Kempen, T. M. G.; Braekman, J. C. (eds), Balkema, Rotterdam. 1994. pp427-440.

[6] Bergmann, W.; Feeney, R. J. *J. Org. Chem.*, **1951**, *16*, 981-987.

[7] Cragg, G. M.; Newman, D. J.; Weiss, R. B. *Seminars in Oncology*, **1997**, *24(2)*, 156-163.

[8] Munro, M. H. G.; Luibrand, R. T.; Blunt, J. W. In: *Bioorganic Marine Chemistry*, Scheuer, P. J. (ed), Verlag Chemie, Berlin, 1987. Vol 1, pp93-176.

[9] Schmitz, F. J. In: *Sponges in Time and Space*, van Soest, R. W. M.; van Kempen, T. M. G.; Braekman, J. C. (eds), Balkema, Rotterdam, 1994. pp485-496.

[10] MarinLit. A marine literature database maintained by the Marine Chemistry Group, University of Canterbury, Christchurch, New Zealand.

[11] Rinehart, K. L.; Gloer, J. B.; Cook, J. C.; Mizsak, S. A.; Scahill, T. A. *J. Am. Chem. Soc.*, **1981**, *103*, 1857-1859.

[12] Schmitz, F. J.; Yasumoto, T. *J. Nat. Prod.*, **1991**, *54(6)*, 1469-1490.

[13] Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.*, **1990**, *55*, 4512-4515. Erratum *J. Org. Chem.*, **1991**, *56*, 1676.

[14] Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55(15)*, 4508-4512.

[15] Pettit, G. R.; Herald, C. L.; Doubek, D. L.; Herald, D. L.; Arnold, E.; Clardy, J. *J. Am. Chem. Soc.*, **1982**, *104*, 6846-6848.

[16] Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinman, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B.; Bontems, R. J. *J. Am. Chem. Soc.*, **1987**, *109(22)*, 6883-6885.

[17] a) Pettit, G. R.; Singh, S. B.; Hogan, F.; Lloyd-Williams, P.; Herald, D. L.; Burkett, D. D.; Clewlow, P. J. *J. Am. Chem. Soc.*, **1989**, *111(14)*, 5463-5465; b) Hamada, Y.; Hayashi, K.; Shioiri, T. *Tet. Lett.*, **1991**, *32*, 931-934; c) Tomioka, K.; Kanai, M.; Koga, K. *Tet. Lett.*, **1991**, *32*, 2395-2398.; d) Shioiri, T.; Hayashi, K.; Hamada, Y. *Tetrahedron*, **1993**, *49*, 1913-1924.; Roux, F.; Maugras, I.; Poncet, J.; Niel, G.; Jouin, P. *Tetrahedron*, **1994**, *50*, 5345-5360.

[18] Uemura, D.; Takahashi, K.; Yamamoto, T.; Katayama, C.; Tanaka, J.; Okumura, Y.; Hirata, Y. *J. Am. Chem. Soc.*, **1985**, *107*, 4796-4798.

[19] Hamann, M. T.; Otto, C. S.; Scheuer, P. J.; Dunbar, D. C. *J. Org. Chem.* **1996**, *61(19)*, 6594-6600. Erratum *J. Org. Chem.*, **1998**, *63(14)*, 4856.

[20] Long, B. H.; Carboni, J. M.; Wasserman, A. J.; Cornell, L. A.; Casazza, A. M.; Jensen, P. R.; Lindel, T.; Fenical, W.; Fairchild, C. R. *Cancer Research*, **1998**, *58*, 1111-1115.

[21] Nicolaou, K. C.; Oshima, T.; Hosokawa, S.; van Delft, F. L.; Vourloumis, D.; Xu, J. Y.; Pfefferkorn, J.; Kim, S. *J. Am. Chem. Soc.*, **1998**, *120*, 8674-8680.

[22] Chen, X.-T.; Gutteridge, C. E.; Bhattacharya, S. K.; Zhou, B.; Pettus, T. R. R.; Hascall, T.; Danishefsky, S. J. *Angew. Chem. Int. Ed. Engl.*, **1998**, *37*, 185-187.

[23] D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta.*, **1987**, *70*, 2019-2027.

[24] Nicolaou, K. C.; Kim, S.; Pfefferkorn, J.; Xu, J.; Oshima, T.; Hosokawa, S.; Vourloumis, D.; Li, T. *Angew. Chem. Int. Ed. Engl.*, **1998**, *37*, 1418-1421.

[25] ter Haar, E.; Kowalski, R. J.; Hamel, E.; Lin, C. M.; Longley, R. E.; Gunasekera, S.; Rosenkranz, H. S.; Day, B. W. *Biochemistry*, **1996**, *35*, 243-245.

[26] Gunasekera, S.; Gunasekera, M.; Schulte, G. K. *J.Org. Chem.*, **1990**, *55*, 4912-4915. Erratum *J.Org. Chem.*, **1991**, *56*, 1346.

[27] Sabbaraju, G. V.; Golakoti, T.; Patterson, G. M. L.; Moore, R. E. *J. Nat. Prod.*, **1997**, *60*, 302-305.

[28] Gardinier, K. M.; Leahy, J. W. *J. Org. Chem.*, **1997**, *62*, 7098-7099.

[29] Smith, C. D.; Zhang, X.; Mooberry, S. L.; Patterson, G. M. L.; Moore, R. E., *Cancer Res.*, **1994**, *54*, 3779-3784.

[30] Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blotchin, A.; Slate, D. L. *J. Org. Chem.*, **1994**, *59*, 1243-1245.

[31] Kobayashi, M.; Aoki, S.; Ohyabu, N.; Kurosu, M.; Wang, W.; Kitagawa, I. *Tet. Lett.*, **1994**, *35 (43)*, 7969-7972.

[32] Talpir, R.; Benayahu, Y.; Kashman, Y.; Panell, L.; Schleyer, M. *Tet. Lett.*, **1994**, *35*, 4453-4456.

[33] a) Petitt, G. R.; Cichacz, Z. A.; Gao, F.; Herald, C. L.; Boyd, M. R.; Schmidt, J. M.; Hooper, J. N. A. *J. Org. Chem.*, **1993**, *58*, 1302-1304; b) Fusetani, N.; Shinoda, K.; Matsunaga, S. *J. Am. Chem. Soc.*, **1993**, *115*, 3977-3981; c) Kobayashi, M.; Aoki, S.; Sakai, H.; Kawazoe, K.; Kihara, N.; Sasaki, T.; Kitagawa, I. *Tet. Lett.*, **1993**, *34*, 2795-2798.

[34] Pietruszka, J. *Angew. Chem. Int. Ed.*, **1998**, *37 (19)*, 2629-2636.

[35] Curtis, H. and Barnes, N. S. *Biology*, Worth Publishers Inc, New York, 1989. 5th edition, pp522-524.

[36] Bergquist, P. R. *Sponges*, Hutchinson & Co. Ltd, London, 1978. 1st edition, p16.

[37] Bergquist, P. R. *Sponges*, Hutchinson & Co. Ltd, London, 1978. 1st edition, p181.

[38] Munro, M. H. G., University of Canterbury, personal communication.

[39] Boyd, M. R. *Principles and Practise of Oncology*, **1989**, *3*, 1-26.

[40] Marston, A.; Décosterd, L. A.; Hostettmann, K. In: *Bioactive Natural Products:Detection, Isolation and Structural Determination*, Colegate, S. M. and Molyneux, R. J. (eds), CRC Press Ltd., Boca Raton, 1993. 1st edition, p222.

[41] Boyd, M. R. In: *Principles and Practise of Oncology Updates*, DeVita, V. T.; Hellman, S.; Rosenberg, S. A. (eds), J. P. Lippincott, Philadelphia, 1989. Vol 3, No. 10, pp1-12.

[42] Boyd, M. R. In: *Current Therapy in Oncology*, Neiderburger, J. E. (ed.), B. C. Decker, Philadelphia, 1992. pp11-22.

[43] Paull, K.; Lin, C. M.; Malspeis, L.; Hamel, E. *Cancer Research*, **1992**, *52*, 3892-3900.

[44] Perry, N. B.; Blunt, J. W.; Munro, M. H. G. *J. Am. Chem. Soc.*, **1988**, *110 (14)*, 4850-4851.

[45] Perry, N. B.; Blunt, J. W.; Munro, M. H. G.; Thompson, A. M. *J. Org. Chem.*, **1990**, *55 (1)*, 223-227.

[46] Perry, N. B.; Blunt, J. W.; McCombs, J. D.; Munro, M. H. G. *J. Org. Chem.*, **1986**, *51 (26)*, 5476-5478.

[47] Perry, N. B.; Blunt, J. W.; Munro, M. H. G. *Tetrahedron*, **1988**, *44 (6)*, 1727-1734.

[48] Perry, N. B.; Blunt, J. W.; Munro, M. H. G.; Higa, T.; Sakai, R. *J. Org. Chem.*, **1988**, *53*, 4127-4128.

[49] Matsunaga, S.; Fujiki, H.; Sakata, D.; Fusetani, N. *Teterahedron*, **1991**, *47*, 2999-3006.

[50] Okada, A.; Watanabe, K.; Umeda, K.; Miyakado, A. *Agric. Biol. Chem.*, **1991**, 2675-2771.

[51] Dumdei, E. J.; Blunt, J. W.; Munro, M. H. G.; Pannell, L. K. *J. Org. Chem.*, **1997**, *62*, 2636-2639.

[52] Li, S.; Dumdei, E. J.; Blunt, J. W.; Munro, M. H. G.; Robinson, W. T.; Pannell, L. K. *J. Nat. Prod.*, **1998**, *61*, 724-728.

[53] Hirata, Y.; Uemura, D. *Pure & Appl. Chem.*, **1986**, *58(5)*, 701-710.

[54] Pettit, G. R.; Herald, C. L.; Boyd, M. R.; Leet, J. E.; Dufresne, C.; Doubek, D. L.; Schmidt, J. M.; Cerny, R. L.; Hooper, J. N. A.; Rützler, K. C. *J. Med. Chem.*, **1991**, *34*, 3339-3340.

[55] Pettit, G. R.; Tan, R.; Williams, M. D.; Doubek, D. L.; Boyd, M. R.; Schmidt, J. M.; Chapuis, J.-C.; Hamel, E.; Bai, R.; Hooper, J. N. A.; Tackett, L. P. *J. Org. Chem.*, **1993**, *58*, 2538-2543.

[56] Pettit, G. R.; Feng, G.; Doubek, D. L.; Boyd, M. R.; Hamel, E.; Bai, R.; Schmidt, J. M.; Tackett, L. P.; Rützler, K. C. *Gazetta Chimica Italiana*, **1993**, *123*, 371-377.

[57] Lake, R. J. Internal Report dated 26/2/88, University of Canterbury.

[58] Bergquist, P. R. *Sponges*, Hutchinson & Co. Ltd, London, 1978. 1st edition, p14.

[59] Litaudon, M.; Hart, J. B.; Blunt, J. W.; Lake, R. J.; Munro, M. H. G. *Tet. Lett.*, **1994**, *35 (50)*, 9435-9438.

[60] Litaudon, M.; Hickford, S. J. H.; Lill, R. E.; Lake, R. J.; Blunt, J. W.; Munro, M. H. G. *J. Org. Chem.*, **1997**, *62*, 1868-1871.

[61] Fodstad, O.; Breistoel, K.; Pettit, G. R.; Shoemaker, R. H.; Boyd, M. R. *J. Exp. Ther. Oncol.*, **1996**, *1(2)*, 119-125.

[62] D. J. Newman, Natural Products Branch, DTP, NCI, USA, personal communication.

[63] Bai, R.; Paull, K. D.; Herald, C. L.; Malspeos, L.; Pettit, G. R.; Hamel, E. *J. Biol. Chem.*, **1991**, *266 (24)*, 15882-15889.

[64] Jordan, A.; Hadfield, J. A.; Lawrence, N. J.; McGown, A. T. *Med. Res. Rev.*, **1998**, *18 (4)*, 259-296.

[65] García-Rocha, M.; García-Gravalos, M. D.; Avila, J. *Br. J. Cancer*, **1996**, *73*, 875-883.

[66] Luduena, R. F.; Roach, M. C.; Prasad, V.; Pettit, G. R. *Biochem. Pharmacol.*, **1993**, *45 (2)*, 421-427.

[67] Page, M. J.; Battershill, C. N. *Mar. Ecol. Prog. Ser.* Submitted for publication.

[68] Blunt, J. W.; Dumdei, E. J.; Hickford, S. J. H.; Lill, R. E.; Li, S.; Munro, M. H. G.; Battershill, C. N.; Duckworth, A. R. *Journal of Biotechnology – Proceedings of the Marine Biotechnology Symposium*, In Press.

[69] Dumdei, E. J.; Blunt, J. W.; Munro, M. H. G.; Battershill, C. N.; Page, M. J. In: *Sponge Sciences. Multidisciplinary Perspectives*, Watanabe, Y.; Fusetani, N. (eds), Springer-Verlag, Tokyo, 1998. pp353-364.

[70] Norcross, R. D.; Paterson, I. *Chem. Rev.*, **1995**, *95*, 2041-2114.

[71] Aicher, T. D.; Buszek, K. R.; Fang, F. G.; Forsyth, C. J.; Jung, S. H.; Kishi, Y.; Matelich, M. C.; Scola, P. M.; Spero, D. M.; Yoon, S. K. *J. Am. Chem. Soc.*, **1992**, *114*, 3162-3164.

[72] Stamos, D. P.; Kishi, Y. *Tet. Lett.*, **1996**, *37*, 8643-8646.

[73] Stamos, D. P.; Chen, S. S.; Kishi, Y. *J. Org. Chem.* **1997**, *62*, 7552-7553.

[74] Munro, M. H. G.; Blunt, J. W.; Lake, R. J.; Litaudon, M.; Battershill, C. N.; Page, M. J. In: *Sponges in Time and Space*, van Soest, R. W. M.; van Kempen, T. M. G.; Braekman, J. C. (eds), Balkema, Rotterdam, 1994. pp473-484.

[75] Garson, M. J. *Unpublished Results.*

[76] Rinkevich, B. *Journal of Biotechnology – Proceedings of the Marine Biotechnology Symposium*, In Press.

[77] Kawamura, O.; Sato, S.; Kajii, H.; Nagayama, S.; Ohtani, K.; Chiba, J.; Ueno, Y. *Toxicon*, **1989**, *27*, 887-897.

[78] Vanderlaan, M.; Watkins, B. E.; Stanker, L. *Env. Sci. Technol.*, **1988**, *182*, 187-192.

[79] Ringsdorf, H. *J. Polymer Sci. Polymer Symp.*, **1975**, *51*, 135-153.

[80] Duncan, R.; Connors, T. A.; Meada, H. *Journal of Drug Targeting*, **1996**, *3*, 317-319.

[81] Duncan, R. *Anti-Cancer Drugs*, **1992**, *3*, 175-210.

[82] Putnam, D.; Kopecek, J. *Adv. Polym. Sci.*, **1995**, *122*, 55-123.

[83] Duncan, R.; Dimitrijevic, S.; Evagorou, E. G. *S. T. P. Pharma Sciences*, **1996**, *6 (4)*, 237-263.

[84] Maeda, H.; Matsumara, Y. *CRC Crit. Rev. Ther. Drug Carrier Sys.*, **1989**, *6*, 193-210.

[85] Duncan, R. *Chemistry & Industry*, **1997**, 263.

[86] Litaudon, M.; Hickford, S. J. H.; Lill, R. E.; Lake, R. J.; Blunt, J. W.; Munro, M. H. G., *J. Org. Chem.*, **1997**, *62*, 1868-1871.

[87] Hart, J. B.; Blunt, J. W.; Munro, M. H. G. *J. Org. Chem.*, **1996**, *61*, 2888.

[88] Hickford, S. J. H., University of Canterbury, personal communication.

[89] Bergquist, P. R.; Tizard, C. A. In *The Encyclopedia of Marine Resources*, Firth, F. E. (ed), 1969. p669.

[90] Duckworth, A. R.; Battershill, C. N.; Bergquist, P. R. *Aquaculture*, **1997**, *156*. 251-267.

[91] Blunt, J. W.; Calder, V. L.; Fenwick, G. D.; Lake, R. J.; McCombs, J. D.; Munro, M. H. G.; Perry, N. B. *J. Nat. Prod.*, **1987**, *50 (2)*, 290-292.

[92] Battershill, C.; Page, M. *Aquaculture Update*, **1996**, 5-6.

[93] Landsteiner, K. *The specificity of seriological reactions*, Dover, New York, 1962.

[94] Grothaus, P., Hawaii Biotechnology Group Inc., personal communication.

[95] Hart, J. B. *PhD Thesis*, **1995**, University of Canterbury.

[96] Uemura, D. In *Bioorganic Marine Chemistry*, Scheuer, P. J. (ed), Springer-Verlag, Berlin, 1991. Vol 4, p13.

[97] Fang, F. G.; Kishi, Y.; Matelich, M. C.; Scola, P. M. *Tet. Lett.*, **1992**, *33 (12)*, 1557-1560.

[98] Uemura, D.; Takahashi, K.; Yamamoto, T.; Katayama, C.; Tanaka, J.; Okumura, Y. and Hirata, Y. *J. Am. Chem. Soc.*, **1985**, *107*, 4796.

[99] Greene, T. W.; Wuts, P. M. *Protective Groups in Organic Synthesis*, Wiley, New York, 1991. 2nd edition.

[100] Nicolaou, K. C.; Pavia, M. R.; Seitz, S. P. *J. Am. Chem. Soc.*, **1981**, *103*, 1224-1226.

[101] Evans, D. A.; Kaldor, S. W.; Jones, T. K.; Calrdy, J. *J. Am. Chem. Soc.*, **1990**, *112*, 7001-7031.

[102] Takai, K.; Hotta, Y.; Oshima, K.; Nozaki, H. *Tet. Lett.*, **1978**, *19*, 2417-2420.

[103] Furber, M.; Mander, L. N.; Patrick, G. L. *J. Org. Chem.*, **1990**, *55*, 4860-4870.

[104] Lombardo, L. *Tet. Lett.*, **1981**, *23*, 4293-4296.

[105] Tebbe, F. N.; Parshall, G. W.; Reddy, G. S. *J. Am. Chem. Soc.*, **1978**, *100*, 3611-3613.

[106] Pine, S. H.; Zahler, R.; Evans, D. A.; Grubbs, R. H., *J. Am. Chem. Soc.*, **1980**, *102*, 3270-3272.

[107] Reetz, M. T. *Organotitanium Reagents in Organic Synthesis*, Springer-Verlag, Berlin, 1986. pp226-227.

[108] Peterson, D. J. *J. Org. Chem.*, **1968**, *33*, 780-784.

[109] Johnson, C. R.; Shanklin, J. R.; Kirchhoff, R. A. *J. Am. Chem. Soc.*, **1973**, *95*, 6462-6463.

[110] Mash, E. A.; Math, S. K.; Flann, C. J. *Tetrahedron*, **1989**, *45*, 4945-4950.

[111] Takai, K., Kakiuchi, T., Kataoko, Y., Utimoto, K. *J. Org. Chem.*, **1994**, *59*, 2668-2670.

[112] Perrin D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, 1980. 2nd edition, p547.

[113] Strohalm, J.; Kopecek, J. *Angew. Makromol. Chem.*, **1978**, *70*, 109-118.

[114] Dr Frances Searle, School of Pharmacy, University of London, UK, personal communication.

[115] Duncan, R.; Hume, I. C.; Yardley, H. J.; Flanagan, P. A.; Ulbrich, K.; Subr, V.; Strohalm, J. *Journal of Controlled Release*, **1991**, *16*, 121-126.

[116] Duncan, R.; Kopecek, J. *Adv. Polymer Sci.*, **1984**, 57, 51-101.

[117] a) Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.*, **1971**, *93 (12)*, 2897-2904; b) Lane, C. F. *Synthesis*, **1975**, 135-146.

[118] Dess, D. B.; Martin, J. C. *J. Org. Chem.*, **1983**, *48*, 4155-4156.

[119] House, D., University of Canterbury, personal communication.

[120] Scriven, E. F. V.; Turnbull, K. *Chem. Rev.*, **1988**, *88 (2)*, 351-368.

[121] Pilard, S.; Vaultier, M. *Tet. Lett.*, **1984**, *25*, 1555-1556.

[122] Gartiser, T.; Selve, C.; Delpuech, J.-J. *Tet. Lett.*, **1983**, *24 (15)*, 1609-1610.

[123] Pretsch, E.; Seibl, J.; Simon, W.; Clerc, T. *Tables of spectral data for structure determination of organic compounds*, English translation of 2nd Ed. 1981, 1983, Springer-Verlag, Berlin, H5.

[124] Corey, E. J.; Nicolaou, K. C.; Balanson, R. D.; Machida, Y. *Synthesis*, **1975**, 590-591.

[125] Krebs, K. G.; Heusser, D.; Wimmer, H. *Thin Layer Chromatography, A Laboratory Handbook, 2nd Edition*, Stahl, E. (ed), Springer-Verlag, New York, 1969. pp873-874.

[126] Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.*, **1978**, *43(14)*, 2923-2925.

[127] Evans, M. B.; Dale, E. D.; Little, C. J. *Chromatographia*, **1980**, *13*, 5-10.

[128] Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*, John Wiley and Sons, Inc, New York, 1967. vol 1, pp1179-1180.